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Interactions of human immunodeficiency virus type 1 with mucosal epithelial surfaces and *Candida albicans*

Islam, Ayesha

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**Interactions of human immunodeficiency virus type 1 with
mucosal epithelial surfaces and *Candida albicans***

Ayesha Islam

**Thesis submitted for the degree of
Doctor of Philosophy**

**Oral Immunology
Clinical and Diagnostic Sciences Group King's
College London**

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Abstract

Despite the magnitude of the HIV pandemic, the events involved in the initial HIV-1 entry into the body are not yet fully understood. Although the principle mode of HIV-1 transmission is through mucosal surfaces, the oral epithelium appears to be less susceptible to HIV-1 infection than vaginal epithelium. In addition, infections with co-pathogens that elicit immune activation, such as *Candida albicans*, may also promote HIV-1 infection.

The project objectives are to determine whether (i) HIV-1 is able to bind and integrate into oral and vaginal epithelial cell lines, (ii) epithelial cell lines are able to transfer viable virus from their surface to permissive cells, (iii) epithelial cells are responding to HIV-1 with changes in intracellular signalling or gene expression profiling, and (iv) *Candida albicans* can affect epithelial susceptibility to HIV-1 or whether it can bind and/or transfer HIV-1 to permissive cells.

We demonstrate that oral, oro-pharyngeal and vaginal epithelial cell lines do not express canonical receptors for HIV-1 but they do express other receptors known to promote HIV-1 binding, including GalCer and syndecan-1. Oral and vaginal epithelial cell models can capture HIV-1, which subsequently does not appear to integrate into the epithelial genome. Therefore, viral replication is not supported. Notably, HIV-1 captured on the epithelial surface remains infectious and can be transferred to permissive cells. Furthermore, like epithelial cell lines, *C. albicans* can also directly bind and transfer HIV-1 to permissive cells. The carbohydrate moieties chitin and β -glucan appear to play a role in mediating viral binding. Notably, transfer of HIV-1 to permissive cells occurs from chitin but minimally from β -glucan. This indicates that fungal-viral interactions may occur at mucosal surfaces that potentially promote HIV-1 infection.

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Abbreviations

HIV-1	Human immunodeficiency virus
AZT	Azidothymidine, also called zidovudine
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
qPCR	Quantitative real time polymerase chain reaction
CCR5	Chemokine (C-C motif) receptor 5
CXCR4	Chemokine (C-X-C motif) receptor 4
T _m	Melting temperature
CD4	T-cell surface antigen T4/Leu-3, T-cell surface glycoprotein CD4
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
ICAM-1	Intercellular adhesion molecule 1
SDC-1	Syndecan-1
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
CPE	Cytopathic effect
DAVID	Database for Annotation, Visualization and Integrated Discovery
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
ARRRP	AIDS Research and Reference Reagent Program
SFM	Serum free medium
P/S	Penicillin/Streptomycin
FCS	Foetal calf serum
DMEM10	Dulbecco's Modified Eagle Medium (4.5 g/l glucose and L-glutamine) with 10% FCS, 1% P/S

PBMC	Primary blood mononuclear cells (equivalent to lymphocytes)
NHOK	Normal human oral keratinocytes
PGEC	Primary genital epithelial cells
TNF	Tumour necrosis factor
TRAIL	TNF related apoptosis inducing ligand
FasL	Fas ligand
h	Hours
min	Minutes
mL	millilitre
μL	microliter

Chapter 1 Introduction

1.1 The HIV epidemic

According to the World Health Organization in 2009, 33.3 million people were living with human immunodeficiency virus (HIV) worldwide with an estimated 2.6 million new infections that year (United Nations Program on HIV/AIDS, 2010). Despite the magnitude of this global pandemic, the precise events involved in initial HIV entry to the body are not yet properly characterised (Shattock et al., 2008). Although the principle mode of HIV transmission in humans is through exposure to mucosal surfaces, the interaction of mucosal epithelial cells with HIV is not fully understood (Shattock et al., 2008; Wu et al., 2003).

Although the total number of HIV positive people in the world appears to be on the decrease, there are still significant areas of concern with respect to transmission of the virus that causes acquired immunodeficiency syndrome (AIDS). At the moment, heterosexual transmission is the most common (Hladik and McElrath, 2008) with women and girls bearing the greatest burden of infection, accounting for more than 50% of people living with AIDS and 76% of young people (aged 15–24 years) living worldwide with HIV (United Nations Program on HIV/AIDS, 2010). A greater risk for transmission is associated with receptive intercourse for men (Jaffe et al., 1985) and women (United Nations Program on HIV/AIDS, 2010). Mother to child transmission is decreasing due to preventative pre-natal drug regimens and the knowledge that there is an oral transmission risk for breast feeding infants. However, intravenous drug users still lack access to sterile needles (United Nations Program on HIV/AIDS, 2010). Men who have sex with men have a staggeringly high burden in Sub-Saharan Africa with greater than 40% infection in the South African and Kenyan capitals (United Nations Program on HIV/AIDS, 2010). Thus, understanding the mechanisms of mucosal infection is critical in continuing the management of the epidemic.

1.2 HIV-1 genomic structure

HIV-1 is a retrovirus consisting of approximately 10 kilobases (Kb) of genetic material encoding nine genes (Figure 1.1). These genes are: *gag* encoding structural proteins, *pol* encoding viral enzymes, *env* encoding gp120 and gp41, and six genes coding for regulatory and accessory proteins *tat*, *rev*, *nef*, *vpu*, *vpr* and *vif* (Freed, 2004; Komano et al., 2005; Trkola, 2004).

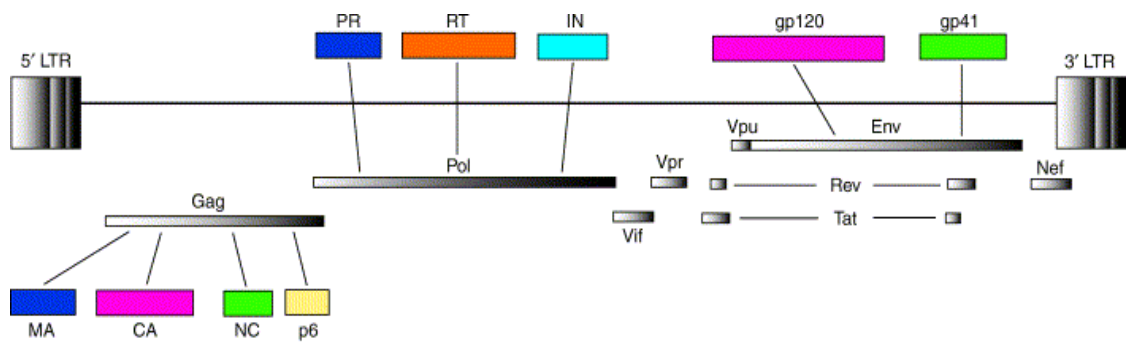
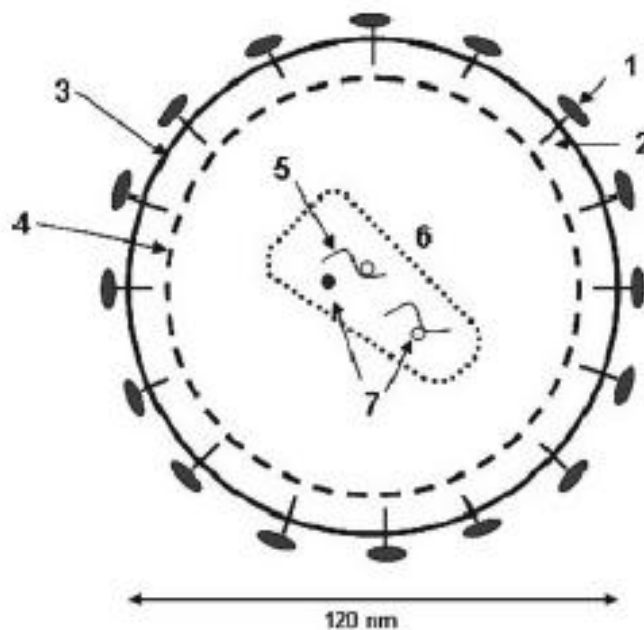


Figure 1-1: Organization of the HIV-1 genome. The location of the long terminal repeats (LTRs) and the genes encoded by HIV-1 are indicated: *Gag*, *Pol*, *Env*, *Vpr*, *Vif*, *Vpu*, *Rev*, *Tat*, *Nef*. Polyproteins Gag, Pol and Env are cleaved by protease digestion. Pol protein is cleaved to form PR: viral protease, RT: reverse transcriptase p51 and p66 and IN: integrase. Env is cleaved to form envelope gp120 and gp41 that form heterotrimers in the virus envelope. Gag is cleaved to form MA: matrix, CA: p21 capsid, NC: nucleocapsid p6 and p7. The sizes of the genes and encoded proteins are not to scale (schematic from Freed, 2004).



1. Env gp120
2. Env transmembrane protein gp41
3. Cell derived lipid bilayer
4. Matrix proteins p17
5. Two single stranded RNA HIV genomes
6. p24 capsid
7. Viral proteins: protease p10, integrase p32, reverse transcriptase p66 and p51 subunits and nucleocapsid protein p7.

Figure 1-2: HIV virion structure. Diagram from (Dandekar et al., 2008). Note that envelope gp120 and gp41 are in hetero-trimeric conformation in the membrane of the virus particle and that approximately 14 of these virus spikes exist on a typical particle (Zhu et al., 2006).

Polyproteins Gag, Pol and Env are cleaved by proteases in order to form viral particles. Gag polyprotein encompasses the viral capsid protein p24 (Figure 1.2, 6.), the nucleocapsid p6 and p7 (Figure 1.2, 7.) and the matrix p17 (Figure 1.2, 4.). Pol is processed to release the viral protease, reverse transcriptase and integrase enzymes (Figure 1.2, 7.). Reverse transcriptase is an error prone RNA-dependant DNA polymerase that is required to convert the HIV RNA genome to complementary DNA that

can be trimmed and integrated into the host DNA genome by the integrase enzyme (Dandekar et al., 2008). The viral envelope comprises a host cell phospholipid bilayer that is gathered from the infected cell as the virus is released (Figure 1.2, 3.). The viral envelope polyprotein gp160 is highly glycosylated as it is folded in the endoplasmic reticulum (ER), the glucose residues are trimmed in the ER and *cis*-golgi, resulting in heterogeneously sized high mannose structures as it moves further down the secretory pathway and finally glucose transferases act in the *trans*-golgi, producing complex outer branching of the sugars (Fenouillet et al., 1995). Gp160 is processed by cellular proteases to form gp120 (Figure 1.2, 1.) and gp41 (Figure 1.2, 2.) that remain non-covalently associated and form a hetero-dimers in trimeric conformation in the virion membrane surface (Chan et al., 1998). Over half the molecular weight of gp120 ends up composed of sugars (Allan et al., 1985; Checkley et al., 2011) : this glycosylation is not necessary for its function but rather its proper folding for biological activity (Fenouillet et al., 1994). A conformational change in gp120 on binding CD4 results in gp41 coming into close contact with the cell membrane and mediates membrane fusion (Chan et al., 1998).

The virion also contains two regulatory proteins. Tat is the “trans-activator of transcription” and regulates HIV transcription by binding the transactivation response element (TAR) in the HIV-LTR (long terminal repeats), the HIV promoter. Tat promotes elongation of full length transcripts (Dandekar et al., 2008; Flint and American Society for Microbiology., 2009) by its interaction with P-TEFb. The P-TEFb protein kinase complex then stimulates elongation by phosphorylating the negative elongation factor (NELF), and similarly activates the polymerase activity by phosphorylation of RNA Polymerase II and Spt5 (Karn, 2011). Rev is the regulator of the virion and binds the Rev-response element (RRE) in env RNA and mediates its nuclear export. It facilitates the production of viral structural proteins and accumulates to promote the late phase of the viral replication cycle (Dandekar et al., 2008).

Four accessory proteins of HIV have been described, so called because they are not essential for the virus life cycle but increase the virus' fitness. Vif, or viral infectivity factor, accumulates in infected cells and increases infectivity by targeting cellular anti-viral APOBEC3G for degradation. Nef or negative regulatory factor has many functions including down regulating CD4 (see section 1.4) and major histocompatibility complex class I surface expression. Vpr or viral protein R may facilitate nuclear import of the HIV pre-integration complex and promotes cell cycle arrest. Vpu is viral protein U and is required for the incorporation of a single viral core in budding (Dandekar et al., 2008; Flint and American Society for Microbiology., 2009).

1.3 HIV-1 Classification

There are four main groups of HIV-1 arising from 4 transmission events of zoonoses from non-human primates to humans : M “major”, O “outlier”, N “new” (Dandekar et al., 2008) and P “preliminary” (Plantier et al., 2009). The majority of HIV-1 (99.6%) infections are of the M group (Kandathil et al., 2005).

The clades or subtypes of group M are A1, A2, B, C, D, F1, F2, G, H, J, K and some circulating recombinant forms (CRF) represent different genetic lineages of HIV-1 and are associated with certain geographic regions (Baeten et al., 2007; HIV database, 2012; McCutchan, 2006). Although clades were originally defined based on the sequence of env and gag genes, these are now classified by comparing whole virus sequences in order to establish the genetic phylogeny of the queried virus (Robertson et al., 2000). CRF arise when a cell is infected with two subtypes of HIV-1. If two viruses infect the same cell, then virions can be packaged containing one single-stranded RNA from each infecting subtype, since two copies of single stranded RNA comprise the core genetic material. When reverse transcription occurs, the viral reverse transcriptase may switch between the two RNA templates, resulting in the creation of a mosaic virus by the dual virally infected cell (HIV database, 2012). In addition, there are strict criteria for the definition of a CRF: the CRF must be sequenced in full and must be found in three or more individuals that are not linked epidemiologically (Hemelaar et al., 2011). Unique recombinant forms (URF) are recombinant viruses in the population that do not fit the criteria for CRF. Thus, there are many forms of HIV-1. This diversity has a great impact on diagnostics and prognosis for the virus carriers, since subtype may affect the rate of disease progression (Baeten et al., 2007; Kiwanuka et al., 2008). In addition, this implies that any vaccine would need to be group and clade specific as well as directed against the most conserved portions of viral genes and proteins. HIV has a very high mutation and recombination rate (Rambaut et al., 2004). Furthermore, the characteristics of the immune systems of the human populations receiving the vaccine will also affect the efficacy of the vaccine. Thus, vaccine development will be challenging.

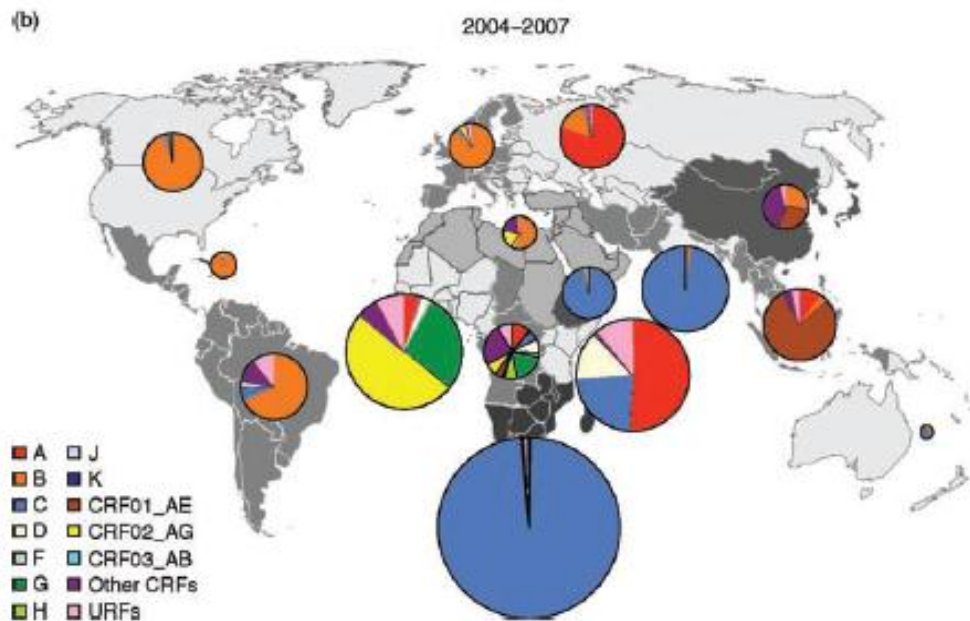


Figure 1-3: Global distribution of subtypes or clades of HIV-1. The world is divided into 15 geographic regions that appear in the same shade of grey. The overlaid pie charts have a surface area proportional to the number of people infected with HIV in the region. The colours represent the subtypes, CRFs and URFs (Hemelaar et al., 2011).

1.4 Classical cycle of HIV-1 infection

The canonical course of HIV-1 infection first requires binding of HIV-1 to permissive cells expressing the CD4 receptor and a chemokine co-receptor (CCR5 or CXCR4). This results in a conformational change in envelope heterotrimers: gp120 and exposes regions of gp41 that are needed for fusion of the virus particle to the cell membrane. Entry of viral capsid containing the viral RNA is followed by uncoating of the virus and reverse transcription of the viral RNA into complementary DNA (Figure 1.4) (Turner and Summers, 1999). Double stranded retroviral circular DNA is imported into the nucleus as part of the viral pre-integration complex (composed of Vpr, viral matrix and integrase that contains a nuclear localisation signal) and integrated into the host genome by viral integrase (Dandekar et al., 2008). These two key stages (reverse transcription and integration) are targeted by highly active anti-retroviral therapy (HAART) for the treatment of HIV and AIDS patients. For example, Azidothymidine (AZT) is a chemical analogue of thymidine acting as a reverse transcription inhibitor, and integrase inhibitors (e.g. 4-[3-(azidomethyl)phenyl]-2-hydroxy-4-oxo-2-butenoic acid) target HIV-1 genome integration into newly infected cells. Transcription is regulated by the long terminal repeat (LTR) sequence of the proviral DNA and the viral regulatory proteins Tat, Rev and Nef are the first proteins to be produced in the early phase of infection. The accumulation of Tat and Rev increases transcriptional activity and initiates the late phase of infection (Sierra et al., 2005). Late phase transcription allows viral

structural proteins to be synthesized. This is followed by glycosylation of envelope proteins, transport, assembly of the virion: packaging the viral genomic RNA at the cell membrane in lipid raft rich areas (Dandekar et al., 2008). Ultimately budding from the infected cell involves the cleavage of Gag and Gag-Pol polyproteins (Hladik and McElrath, 2008).

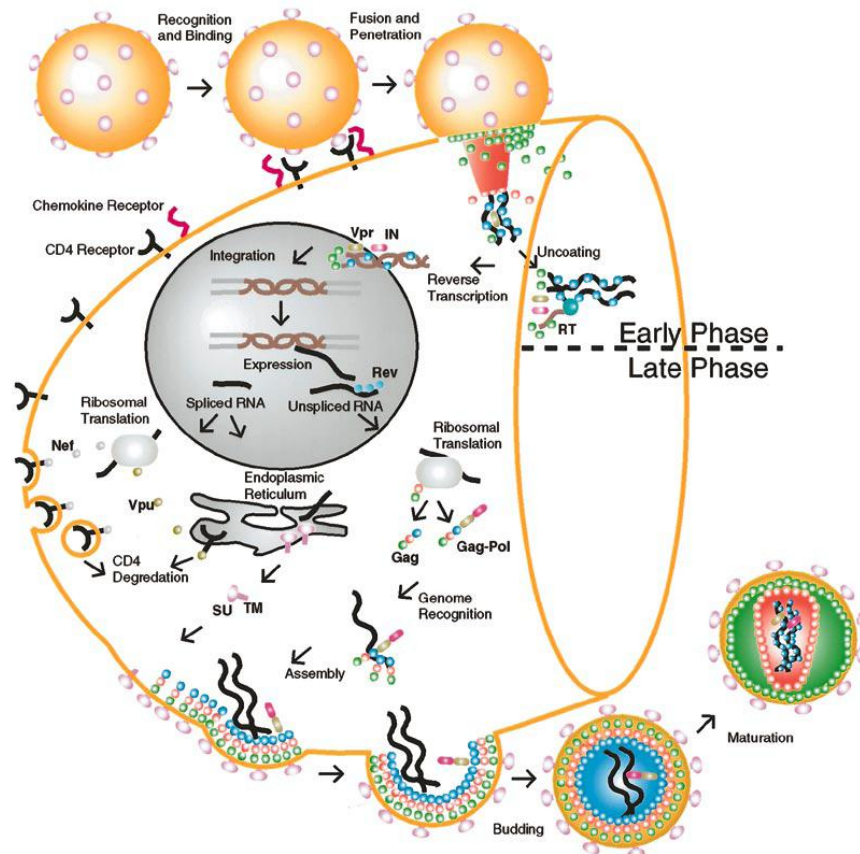


Figure1-4: HIV-1 Life Cycle. General features of canonical HIV-1 infection. The early phase begins with CD4 recognition and involves events up to and including integration of the proviral DNA: gp120 is bound by CD4 and recruits the chemokine receptor CXCR4 or CCR5. The virus and cell membranes fuse and the viral DNA is uncoated, reverse transcribed and integrated into the host DNA. The virus can remain in a latent state (for an indeterminate amount of time) or proceed to the late phase. The late phase comprises transcription of the integrated DNA, translation of accessory proteins, virus assembly, virus budding and maturation. Figure from Turner and Summers, 1999.

HIV-1 establishes and conceals itself in the Gut Associated Lymphoid Tissue (GALT), CD4⁺ T cells, macrophages, dendritic cells and B-lymphocytes. HIV-1 can also infect endothelial cells and affects the central nervous system, reducing the number of CD4⁺ T cells until cellular mediated immunity is lost. HIV-1 infection progresses over time (Figure 1.5) from an acute infection where flu-like symptoms and a spike in viral load occur, to a chronic HIV infection (associated with co-infections) and,

finally, to AIDS where the immune system collapses due to the large scale death of CD4⁺ T cells. This allows potentially fatal infection by opportunistic pathogens and cancers (Flint and American Society for Microbiology., 2009).

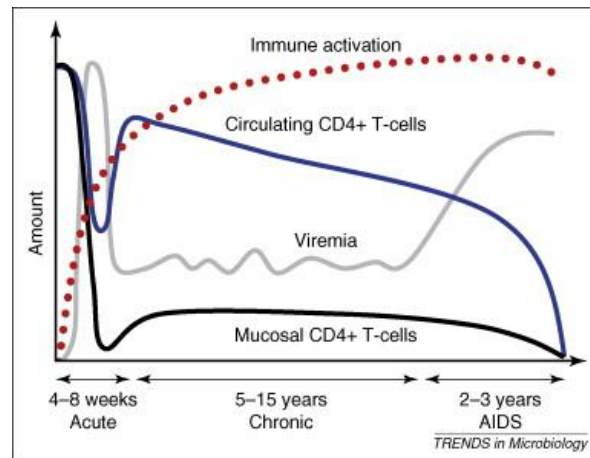


Figure 1-5: Typical course of HIV infection. Increase in immune activation (red), spiking of CD4⁺ T-cells (blue, circulating; black, mucosal) and their decline as well as early spike in viremia that decreases, then fluctuates and finally increases dramatically (grey) (Forsman and Weiss, 2008).

1.5 Receptors and co-receptors

Canonical HIV-1 infection is initiated through binding of the trimeric envelope heterodimers to CD4 that then recruit the chemokine co-receptors CCR5 or CXCR4 (Flint and American Society for Microbiology., 2009). Viruses that are designated R5 trophic use the CCR5 co-receptor for entry to monocytes and macrophages (Alkhatib et al., 1996; Deng et al., 1996), while X4 trophism refers to the virus' use of CXCR4 as a fusion co-receptor to CD4⁺ T cells (Feng et al., 1996). Dual trophic viruses also exist that can utilize either co-receptor with CD4. R5 viruses tend to be first to establish the infection while X4 emergence appears to be associated with clinical deterioration (Xiao et al., 2000). CD4, CCR5 and CXCR4 have naturally occurring ligands: IL-16, β -chemokines (RANTES, MIP-1 α and MIP-1 β) and SDF-1, respectively (Flint and American Society for Microbiology., 2009). Binding of CD4 to gp120 triggers a conformational change in envelope heterotrimers to reveal gp41 which mediates membrane fusion (Gonzalez-Scarano et al., 1987).

1.6 Non-canonical HIV-1 receptors or binding factors

Many alternative co-receptors for HIV-1 entry exist. The glycosphingolipid galactosylceramide (GalCer) has been proposed as an alternative receptor for HIV-1 in CD4 negative cell types (Cook et al., 1994; Fantini et al., 1993; Yahi et al., 1992).

Syndecans are the most common type of heparan sulfate proteoglycan (HSPG) on eukaryotic cells (Bobardt et al., 2003; de Witte et al., 2007) and include Syndecan-1 (SDC-1) which binds HIV Tat and increases endothelial cell movement, and Syndecan-4 (SDC-4) which forms complexes with CXCR4 in human primary lymphocytes, macrophages and HeLa cells. Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) also has an affinity for HIV-1 and can enhance *trans* infection of dendritic cells (Geijtenbeek et al., 2000). There is the mannose receptor that binds HIV-1 (Turville et al., 2002; Cardona-Maya et al., 2006; Nguyen and Hildreth, 2003; Trujillo et al., 2007) through gp120 on vaginal epithelium (Fanibunda et al., 2011). In addition, other C-type lectins other than DC-SIGN and mannose receptor have been shown to have a role in mediating HIV's interaction with Dendritic cells (such as Langerin) (Turville et al., 2002). Two papers specifically implicate the human scavenger receptor gp340 on female genital tract epithelium in facilitating the transcytosis of HIV-1 (Stoddard et al., 2007; 2009). β -1 integrin has also been implicated (Maher et al., 2005). Equally, α 4 β 7 integrin was identified as an env binding factor in T-cells (Arthos et al., 2008) but recent work suggests that blocking this molecule doesn't impair transmission of founder and chronic subtype C HIV-1 (Parish et al., 2012). However, interaction of HIV-1 with alternative receptors may not necessarily lead to fusion and active entry into cells. Interaction instead could promote association with the entry receptors in *cis* and in *trans*. These molecules might thereby protect bound virus from degradation or passively allowing its dissemination in the body through viral presentation to susceptible immune target cells below the mucosal surface (Bobardt et al., 2003; Saidi et al., 2007).

1.7 HIV-1 and the epithelium

Vaginal transmission of the virus is facilitated when epithelial barriers are breached: *ex vivo*, HIV-1 can quickly infect intraepithelial vaginal Langerhans cells and CD4⁺ T-cells when the epithelial barrier is damaged by chemical treatment (Hladik et al., 2007). However, in nature, transmission of cell-free or cell-associated viral particles may occur via several mechanisms, including (1) epithelial breach by micro-tears in the epithelium, (2) the attraction of immune cells to the epithelial lumen by inflammatory signals, and (3) passive transcytosis (Hladik and McElrath, 2008), 4) transmigration of infected cells (Anderson et al., 2010; Shattock et al., 2003). Host cells that could potentially be infected by HIV-1 are vaginal epithelial cells (Asin et al., 2004; Asin et al., 2003; Tan et al., 1993), intraepithelial CD4⁺ T cells, dendritic cells and macrophages (Hladik and McElrath, 2008).

Although normal human oral keratinocytes and oral epithelial cells may be susceptible to productive infection by HIV-1 *in vitro* (Liu et al., 2003; Moore et al., 2002;

Moore et al., 2003; Vacharaksa et al., 2008), others dispute this finding (Quinones-Mateu et al., 2003), while one study found that HIV-1 is inactivated when it passes through adult oral epithelium (Tugizov et al., 2011). However, the fact that oral transmission is seldom reported *in vivo* (Challacombe and Naglik, 2006; Rothenberg et al., 1998) fuels the debate.

Greenhead et al. (2000) used primary cervical and vaginal explants in order to shed light on the nature of the cells that were the primary targets of HIV in the genital epithelium. Although immune activation, as modelled by PHA and IL-2 treatment, in the genital mucosal explants appeared to predispose to greater accumulation of p24, the cells positive for p24 in the explants were also CD68 positive, a marker for macrophages and monocytes (Greenhead et al., 2000). The epithelial cells themselves did not appear to become infected or to foster transcytosis of the the two HIV strains tested (Greenhead et al., 2000).. However, more recent work in primary female genital tract epithelium disputes that epithelial cells are not involved in transcytosis of HIV and implicate HSPG (Bobardt et.al., 2007; Stoddard et al., 2009) and gp340 in the active traversal of HIV-1 through the mucosal barrier (Stoddard et al., 2009).

Thus, a systematic review of the data is required. Firstly, mRNA and protein expression of HIV receptors and related co-factors in and on oral and vaginal epithelium and HIV-1 binding to epithelium will be explored. Next, experimental outcomes of HIV-1 infection and transfer to susceptible cells will be examined. Finally, epithelial responses to HIV-1 and HIV-1 in combination with co-infections will be reviewed.

1.7.1 Canonical and non-canonical receptor expression in epithelium

1.7.1.1 mRNA expression

1.7.1.1.1 Oral epithelium

Currently, there is mRNA expression data on primary human salivary gland epithelial cells, oral keratinocytes, gingival and adenoid epithelial cells in the literature that are summarized in Table 1.1. However, the data is often semi-quantitative as shown by the intensity of an RT-PCR band. In 2002, Moore *et al.* examined two salivary gland epithelial lines; both were negative for *CD4* but positive for *CXCR4*. One of the two lines expressed *CCR5* (Moore et al., 2002). This was closely followed by another publication characterizing two keratinocyte and two adenoid epithelial cell lines (Moore et al., 2003). All four lines expressed *CCR5* and *CXCR4* while only the adenoid epithelial cells expressed *CD4* (Moore et al., 2003). The study by Acheampong *et al.* only showed quantitative RNA data for primary human oral keratinocytes in terms of fold increase in expression when treated with HIV-1 gp120 or Nef or Tat (Acheampong et al., 2005).

Therefore, resting levels of expression were not detailed, preventing comparison of this data set with the other studies.

Giacaman *et al.*, in a study on the effect of *Porphyromonas gingivalis* on oral keratinocytes showed (using an agarose gel), that in unstimulated TERT-2 cells, that *CXCR4* and to a lesser extent *CCR5* were expressed in this immortalized human oral keratinocyte line (Giacaman *et al.*, 2007). Also, Moutsopoulos *et al.* used seven gingival keratinocyte biopsies in a microarray to show that these samples were weakly positive for *CD4*, *CCR5* and *CXCR4* as well as having expression of *SDC-1* and low levels of *DC-SIGN* (Moutsopoulos *et al.*, 2007). However, no unit for the level of expression was given in this study, making it difficult to evaluate this work.

Thus, mRNA expression appears to depend on the type of oral epithelial cell studied (see summary in Table 1.1). Some oral epithelial cells appear to express *CD4*, although not the majority. However, many express at least a small amount of one or both HIV-1 co-receptors (*CCR5* or *CXCR4*). Data pertaining to mRNA expression of non-canonical HIV-1 binding factors is limited. Thus, there needs to be more elucidation of quantitative mRNA expression of HIV-1 receptor related genes in oral epithelial cells.

Table 1-1: HIV-1 receptor related mRNA expression in oral epithelial cells

Type of epithelium	<i>CD4</i>	<i>CXCR4</i>	<i>CCR5</i>	Reference
Salivary gland HSY	-	+	+	Moore <i>et al.</i> 2002
Salivary gland HSG	-	+	-	
Gingival Keratinocyte A	+	+	+/-	Moore <i>et al.</i> 2003
Gingival Keratinocyte B	+	+	+/-	
Adenoid isolate A	-	+	+	
Adenoid isolate B	-	+	+	
Primary Oral Keratinocyte	Not able to determine from publication: comparative microarray data only			Acheampong <i>et al.</i> 2005
TERT-2 (floor of the mouth)	n/a	+	+/-	Giacaman <i>et al.</i> 2007
Gingival Keratinocytes (7 samples)	+/-	+/-	+/-	Moutsopoulos <i>et al.</i> 2007 from microarray data
Tonsil epithelium (5 samples)	+/-	+	+/-	

+: expressed

+/-: low but detectable

-: not expressed
n/a: not applicable
A and B represent different isolates

1.7.1.1.2 Oro-pharyngeal epithelial cells

Few studies have examined mRNA expression of HIV-1 receptor related genes in pharyngeal cells. Moutsopoulos *et al* (2007) have shown microarray data expression levels for five tonsil epithelium samples in comparison with gingival samples. The tonsil samples expressed *CD4* and more *CXCR4* and *CCR5* as compared to the gingival biopsies (Moutsopoulos *et al.*, 2007). However, as mentioned above, no expression units for this data were given. Also, in the same study they list gene expression for GalCer (Moutsopoulos *et al* 2007). However, GalCer is not detectable by microarray as it is a non-protein encoding glycosphingolipid - it can only be detected by antibody. This brings the findings of the study into question as the data deals only with mRNA expression rather than surface detection of GalCer by antibody.

1.7.1.1.3 Vaginal epithelial cells

Studies analysing mRNA expression of HIV-1 receptor related genes in vaginal epithelial cells were not found in recent literature.

1.7.1.1.4 Other epithelial sites

To our knowledge, only one study has quantified HIV-1 receptor related mRNA expression in other epithelial cell types. This was undertaken with the HT-29 colonic carcinoma cell line, which appears to be negative for *CD4* expression by Northern blot (Omary *et al.*, 1991).

1.7.1.2 Surface protein expression

1.7.1.2.1 Oral epithelium

Detection of surface-expressed canonical and non-canonical HIV-1 related receptors by flow cytometry is more commonly investigated than detection of mRNA expression, and the data are listed in Table 1.2. However, the results are often presented in a semi-quantitative manner with histograms depicting the fluorescence distribution of the cells for any given molecule. Moore *et al.* showed that two salivary gland epithelial lines expressed surface GalCer and CXCR4 and one expressed a small amount of CCR5 and no CD4 (Moore *et al.*, 2002), while gingival keratinocytes expressed CXCR4, CCR5 and GalCer (Moore *et al.*, 2002; Moore *et al.*, 2003). Liu *et al* (2003) assayed normal human oral keratinocytes by flow cytometry and after two weeks of culture found no CD4 expression, minimal CCR5 expression and some GalCer and CXCR4 expression.

Giacaman *et al.* (2007) showed that the TERT-2 oral keratinocyte line expressed CXCR4 but very low levels of CCR5 on their surface. Vacharaksa *et al* (2008) followed with more quantitative data for tonsil biopsies grown in culture and TERT-2 where they quantified the epithelial marker CD104 β integrin, HSPGs, GalCer, CD4, and HIV-1 co-receptors. Overall, the flow cytometry data showed that the TERT-2 cells were epithelial (83% \pm 4%, using the epithelial marker CD104 β integrin), expressed high amounts of HSPGs (91% \pm 20), <1% of CD4, GalCer and CCR5, and minimal CXCR4 (3.5%). This showed that TERT-2 cells provided consistent data between studies.

Most recently, Tugizov *et al.* (2011) published flow cytometry data on adult tongue and tonsil epithelium versus foetal tongue and oropharyngeal epithelium. Oral epithelial from adult tongue HSC-3^{sort} (immortalised) and ATNG#1, and foetal tongue FTNG#1 will be discussed in this section and the oropharyngeal samples discussed in section 1.6.1.2.3. The authors did not detect CD4 on any of these epithelial cells while HIV-1 co-receptors (on HSC-3^{sort}) and HSPGs were expressed at relatively low levels. However, samples expressed relatively higher levels of GalCer surface protein expression as compared with the other moieties. The number of experiments this data represents is not specified.

1.7.1.2.2 Thus, the overall consensus is that minimal or no CD4, CCR5, CXCR4 are expressed on the surface of oral epithelial cells but this is variable dependant on the exact origin of the cells. RNA expression was overall consistent with the flow cytometry findings. Prior to the Vacharaksa *et al.* (2008) study, only one representative experiment from three or four was shown. However, with this many repeat experiments, it should have been possible to quantify the percentages of positive cells and show error bars or undertake statistical analysis. This would have allowed more straightforward inter paper comparisons. Female genital epithelium

Detection of surface-expressed canonical and non-canonical HIV-1 related receptors by flow cytometry in vaginal epithelium is summarized in Table 1.2. Asin *et al* (2003) examined two uterine epithelial lines (ECC1, RL95-2) and HEC-1A (endometrial) and showed that all three expressed CD4, CXCR4 and minimal GalCer. Saïdi *et al* (2007) studied the HEC-1A line and found high levels of GalCer (contradicting the previous study) and very high levels of CXCR4. Bobardt *et al* (2007) studied primary vaginal genital epithelial cells from four donors showing average values in mean fluorescence units. The cells expressed very low levels of CD4, CXCR4, CCR5 and GalCer but high levels of HSPGs, including SDC-1 and SDC-2. However, with no standard deviations listed, it is difficult to evaluate whether this data set shows agreement between samples or not.

Most recently, Tugizov *et al* (2011) did not detect CD4 on the surface of HEC-1A or cervical biopsy samples (CERV#1). High expression of GalCer was detected on HEC-1A cells, like Saïdi *et al* (2007), with some expression of HSPGs and low CCR5 and CXCR4 expression. The cervical sample, on the other hand, expressed GalCer poorly, with higher CCR5 and CXCR4 expression and similar HSPG levels as compared with HEC-1A cells.

Therefore, overall, the data for uterine and vaginal epithelium suggests that these cell types express low levels of CD4 but higher co-receptor levels, especially in cell lines. They also seem to express the alternate HIV-1 receptor GalCer, again more so on cell lines than primary cells. Therefore, based on the surface-expression of HIV-1 related receptors, it would appear that female genital epithelial cells may have a greater potential to bind HIV-1 than oral epithelial cells.

Table 1-2: HIV-1 receptor related surface expression in epithelial cells by flow cytometry

Epithelial cell type	CD4	CXCR4	CCR5	Gal Cer	HSPG	Epithelial marker	DC-SIGN	Reference Comment
<u>Oral</u> Salivary gland HSY	-	+	+	+	n/a	n/a	n/a	Moore, 2002
HSG	-	+	+/-	+	n/a	n/a	n/a	Moore, 2002
Gingival keratinocytes	n/a	++	++	+	n/a	n/a	n/a	Moore, 2003 Represents 4 analyses
Normal oral keratinocyte	-	+	+	++		epithelial pan control ++	n/a	Liu, 2003 106 NHOKs
TERT-2	n/a	+	+/-	n/a	n/a	n/a	n/a	Giacaman, 2007
TERT-2	< 1%	3.5% (+/-2%)	< 1%	< 1%	91% (+/-20%)	CD104 β integrin 83% (+/-4)	n/a	Vacharaksa, 2008 Mean of 3 expt
HSC-3 ^{sort} immortalised adult tongue epithelial cells	-	+/-	+/-	100	20	n/a	n/a	Tugizov, 2011 (in mean fluorescence intensity)
Adult tongue	-	30	35	70	35	n/a	n/a	
Foetal tongue	-	40	40	70	30	n/a	n/a	

Epithelial cell type	CD4	CXCR4	CCR5	Gal Cer	HSPG	Epithelial marker	DC-SIGN	Reference Comment
<u>Oro-Pharyngeal</u> Tonsil epithelium explant	< 1%	< 1%	< 1%	4% (+/- 0.1)	13% (+/-7)	CD104 β integrin 80% (+/-11)	< 1%	Vacharaksa, 2008 Mean of 4 samples (1 of which run 2x)
Adult tonsil	-	24	30	95	25	n/a	n/a	Tugizov, 2011 (in mfi)
Foetal oropharyngeal	-	35	20	115	50	n/a	n/a	
<u>Female genital tract</u> ECC1 (uterine)	(~20%)	35%	-	20%	n/a	n/a	n/a	Asin, 2003
RL-95-2 (uterine)	(~20%)	20%	-	65%	n/a	n/a	n/a	Asin, 2003
HEC-1A (endometrial)	(~20%)	6%	-	5%	n/a	n/a	n/a	
HEC-1A	-	10	12	130	30	n/a	n/a	Tugizov, 2011
Cervical	-	30	25	40	23	n/a	n/a	
Primary vaginal epithelium	+/- (Less than 40)	+ (~50)	+ (~60)	+ (~45)	+++ (600) ++SDC-1 (~300) ++SDC-2 (~425)	n/a	n/a	Bobardt, 2007 Mean of 4 donors: 10 ⁵ cells each (in fluorescence units)
HEC-1A	-	89% (mfi:39)	0%	91% (mfi: 41)		n/a	n/a	Saïdi, 2007 1 representative of 3 In mean fluorescence intensity
<u>Colorectal</u> HT-29	n/a	91% (mfi:44)	69% (mfi:	94% (mfi: 98)		n/a	n/a	

			19)					(mfi)
<u>HeLa</u>		++	+++	n/a	n/a	n/a	n/a	Giacaman, 2007
TZMbl								
TZMbl	++++	++	+++	n/a	n/a	n/a	n/a	Wen, 2010 supplemental fig 2

++++, +++, ++: Highest to high levels of expression

+: positive surface expression

+/-: low expression levels

-: not detected

n/a: not applicable (not tested)

1.7.1.2.3 Pharyngeal epithelium

Few studies have investigated oropharyngeal cells with regard to surface-expression of HIV-1 related receptors (summarized in Table 1.2). Vacharaksa *et al.* (2008) published quantitative flow cytometry data for tonsil biopsies grown in culture where the epithelial marker CD104 β integrin, HSPGs, GalCer, CD4, and HIV-1 co-receptors were assessed. Overall, the FACS data showed that the tonsil cells were epithelial (80% +/-11%) and expressed lower levels of HSPGs but slightly greater levels of GalCer compared with TERT-2 cells. However, like TERT-2 cells, the tonsil cells expressed <1% CD4 and CCR5. In effect, the tonsil biopsy samples were globally similar to the TERT-2 cells.

Tugizov *et al.* (2011) also examined adult tonsil (ATNSL#1) and foetal tonsil (FORPH#1) cells by flow cytometry. CD4 was not detected in either sample. However, these samples expressed higher levels of GalCer than age matched tongue-derived epithelium. FOROPH#1 was also slightly higher in HSPG than FTNG#1 (Foetal tongue cells).

Therefore, according to these two studies, oropharyngeal epithelium does not appear to express canonical HIV-1 receptors or DC-SIGN, but does express differential levels of GalCer and HSPGs. Therefore, oropharyngeal epithelium may bind HIV-1 despite poor expression of canonical HIV-1 receptors.

1.7.1.2.4 Other types of epithelium

Few studies have investigated the surface-expression of HIV-1 related receptors in other types of epithelium. Colon cancer lines HT29 and HUT78 were shown to be CD4 negative but SW620 tested CD4 positive (Omary *et al.*, 1991). HT-29 colonic epithelium also expresses high levels of CCR5, CXCR4 and GalCer (Saïdi *et al.* 2007).

1.7.1.2.5 Data comparison between epithelial studies

In the studies described above, different data were often found and therefore contradictory conclusions reached, even if the same cell line was used. There are many potential reasons for this but probably the most relevant are utilising cells at a different passage number, minor differences in culture media and growth conditions, and the use of different antibodies (and their differing efficiencies) in detecting surface moieties. The phase of the cell cycle at mRNA isolation or flow cytometry could also alter surface expression. Therefore, many variables can influence the results obtained by any given study.

There is also evidence that the female genital tract has a differing capacity to capture HIV-1 during the menstrual cycle because expression of the canonical receptors may vary with hormonal changes as shown by fluorescence microscopy (Yeaman et al., 2003). CD4 and CCR5 appear to be highly expressed during the proliferative phase, with a concomitant increase in CXCR4 expression. However, during the secretory phase, CD4 and CCR5 expression decrease, while CXCR4 remains elevated. GalCer, on the other hand, is highly expressed during the secretory phase but reduced in the proliferative phase (Yeaman et al., 2003).

Overall, epithelial cells in general tend to express canonical HIV-1 receptors poorly but do express some of the alternate non-canonical receptors for HIV-1, GalCer and HSPGs. Thus, epithelial cells from many mucosal sites could potentially bind HIV-1.

1.7.2 HIV-1 Capture by Epithelium

Some of the early studies gained insight into the interaction between HIV-1 and the epithelium by interfering with HIV-1 binding using antibodies or other molecules. Anti-CD4 antibodies blocked infection of some colonic epithelial lines but not HT29 (Fantini et al., 1991; Omary et al., 1991). However, GalCer peptides that interfered with binding of gp120 to GalCer decreased HIV-1 infection by 50%. In the same study, the gp120 V3 loop was implicated in binding GalCer (Yahi et al., 1995). Next, heparin was shown to compete with 90% of HIV-1 RF (X4) binding as measured by p24 ELISA compared with untreated and D-mannose treated cells (Wu et al., 2003). Enzyme removal of cellular heparin (with heparinase) also reduced HIV-1 binding to uterine epithelial cells as determined by p24 detection (Wu et al., 2003). Later, using HEC-1A endometrial cells stripped of HSPGs, it was shown that the majority of HIV-1 X4 and R5 interactions were abolished (Saidi et al., 2007). In addition, fucose, N-acetylglucosamine and mannosylated-residues inhibited the transcytosis of HIV-1, implicating mannose receptors in the interaction of HIV-1 with epithelium (Saidi et al., 2007). Finally, binding of

HIV-1 to primary vaginal epithelial cells was confirmed to be independent of CD4, CXCR4 and CCR5 using inhibitors and neutralising antibodies (Bobardt et al., 2007).

Other studies have provided physical/visual evidence for an interaction of HIV-1 with epithelium. Particles coated with HIV-1 gp120 were seen to be captured by gingival epithelial cells, suggesting that the virus could be taken up by epithelium (Kage et al., 1998). Correlating this finding, the co-localization of HIV-1 RNA with keratinocytes in oral biopsies from HIV-1 patients was observed (Rodriguez-Inigo et al., 2005).

Methods have been developed for visualising the direct interaction between the epithelial membrane and HIV-1 but these investigations still do not satisfactorily demonstrate whether a direct binding event had occurred. Since the advent of GFP as a molecular label, the HIV field has used this molecule to track virus particles via fusion with the viral protein Vpr, since the protein is packaged in the virus particle. HIV-1 GFP-Vpr was shown to bind to exposed differentiated oral epithelial models (Nittayananta et al., 2009) as well as adult tonsil biopsies (Tugizov et al., 2011) as determined by confocal microscopy. However, it was difficult to visualize distinct virus particles interacting directly with the epithelium. Electron microscopy has also been used to visualize HIV-1 infected peripheral blood mononuclear cell (PBMC) interacting with intestinal (Alfsen et al., 2005) or foreskin epithelial cells (Ganor et al., 2010) but, again, in both studies it was difficult to visualize distinct virus particles attached to the epithelial surface. Amidst this apparent confusion, a reliable and sensitive method to directly visualize HIV-1-epithelial interactions would enhance understanding in the field.

1.7.3 HIV-1 integration and productive infection

HIV-1 infection is difficult to define. As shown in Figure 1.4, there are many steps that are characteristic of infection: binding, reverse transcription, integration, reactivation, replication and particle formation. Productive infection is less ambiguous since it refers specifically to the release of *de novo* made infectious virus particles. Although various epithelial cell types appear to bind and be infected by HIV-1 *in vitro*, consensus states that the oral mucosa is a privileged site resistant to HIV-1 infection. Data exists with respect to whether oral epithelial cells can be infected by HIV-1, but many different cell types have been studied using a variety of conditions with contradictory results and readouts (see Table 1.3). Infection was often measured by p24 accumulation in epithelial culture supernatant or p24 accumulation associated with oral and vaginal cells (Asin et al., 2004; Asin et al., 2003; Han et al., 2000; Moore et al., 2003; Pang et al., 2000). More recently a publication examining the TERT2 immortalized cell line as a surrogate for oral epithelium reported viral integration but abortive replication (Vacharaksa et al., 2008).

However, the integration assay in this study was not carried out in the same manner as described in the proof-of-principle publications to assess HIV-1 integration by this method (Brussel and Sonigo, 2003, 2004). The authors conclude that productive infection occurred in the TERT2 cells because trypsin-treated TERT2 cells could infect susceptible cells (Vacharaksa et al., 2008). However, studies in vaginal epithelium found that trypsin treatment did not remove all virus from the epithelial cell surface as p24 could still be detected on the epithelial cell surface (Dezzutti et al., 2001; Wu et al., 2003). Therefore, the studies demonstrating epithelial p24 accumulation data and HIV-1 transfer may not actually be reporting productive viral infection from

Table 1-3: HIV-1 infection of oral epithelial cells.

Cell type	Reference	Binding, productive infection, transfer of infectious virus
Epithelial cell lines from salivary gland: HSY, HSG	(Han et al., 2000)	Productive infection (p24, provirus in cells and competent to infect T-lymphoblastoid cell line)
Oral carcinoma cell lines: TU139 and TU177. Cervical cancer HeLa and HeLa CD4.	(Pang et al., 2000)	Productive infection leading to EGFP expression (p24, GFP expression blocked by anti-retroviral azidothymidine (AZT) and neutralizing anti-gp120 antibody)
Primary human oral keratinocytes	(Liu et al., 2003)	Productive infection by X4 and dualtrophic virus in presence of polybrene (p24 peak at 10 fold less than infected peripheral blood lymphocyte levels, proviral DNA detection by PCR and could infect activated peripheral blood lymphocytes in co-culture)
Primary human oral epithelial cells	(Quinones-Mateu et al., 2003)	No infection after 5 days exposure by RT activity in culture supernatant and real time PCR for proviral DNA.
Primary human oral keratinocytes	(Acheampong et al., 2005)	Productive infection (p24 over 6 days culture) with polyamines (spermine and spermidine in concentration similar to human semen) and dNTPs.
Epithelial cell lines from salivary gland: HSY, HSG	(Moore et al., 2002)	Cell-associated virus transmission to epithelial cells (from PBMC, CEM T-cell line and monocytes by p24).
Primary human gingival keratinocytes and adenoid epithelial cells	(Moore et al., 2003)	Cell-free R5, but not cell-associated HIV-1, infected gingival keratinocytes (p24). Gp160 anti-sera blocked infection. Infected keratinocytes released infectious virus that could infect PBMC via a cell-free mechanism. Cell-free (X4) and cell-associated (R5) productively infected adenoid epithelial cells.
Human palatine tonsil	(Maher et al., 2005)	Cell-associated infection of primary tonsil epithelial cells by HIV-1 infected lymphocytes (p24 immunohistochemistry increased over time)

Cell type	Reference	Binding, productive infection, transfer of infectious virus
Human palatine tonsil stratified squamous epithelium and cryptal epithelium	(Kumar et al., 2006)	Surface epithelial cells did not support infection (p24)
OKF6/TERT-2 cell line	(Vacharaksa et al., 2008)	Integrated HIV-1 DNA. However, abortive replication: multiply spliced and unspliced HIV mRNAs not detected. Transfer to PBMC or MOLT4 cells.
Human buccal (TR146), pharyngeal (FaDu) epithelial cell lines, vulval (A431)	Kohli, A and Islam, A (submitted manuscripts, and this thesis)	Binding detected by flow and transfer of live virus to susceptible reporter cells. No DNA integration. No productive infection. Productive infection achieved only when endocytic pathway used for entry with a VSV-G envelope.

within the epithelial cells but simply HIV-1 that is captured on the surface of the epithelium during initial exposure and released on contact with susceptible cells.

1.7.4 Transfer to permissive cells

Many studies have described the ability of oral and vaginal epithelium to transfer HIV-1 to susceptible, CD4 expressing cell types (Asin et al., 2004; Asin et al., 2003; Bobardt et al., 2007; Giacaman et al., 2008; Han et al., 2000; Liu et al., 2003; Maher et al., 2005; Moore et al., 2003; Vacharaksa et al., 2008; Wu et al., 2003). However, as discussed above, this is not necessarily reflective of productive infection within the epithelial cell (Dezzutti et al., 2001; Wu et al., 2003).

For oral and pharyngeal epithelial cells many studies have been published. Liu *et al.* (2003) incubated normal human oral keratinocytes (NHOK) with HIV-1 Ba-L (R5) and NL4.3 (X4), washed and then incubated the cells with peripheral blood lymphocytes (PBL) that subsequently became productively infected (Liu et al., 2003). Transmission of HIV-1 from salivary gland epithelial lines, keratinocytes and adenoid epithelial cells to PBMC and CEM T-cell line and monocytes has also been reported (Moore et al., 2002; Moore et al., 2003). Maher *et al.* (2005) used tissue explants derived from tonsillectomies in order to study HIV-1 interacting with the tonsil epithelium and resident immune cells. Firstly, they showed HIV-1 infected T-cells bound to the surface of the epithelium and caused p24 accumulation in explants by immunohistochemistry. Basal epithelial cells were positive for p24, suggesting transmigration of HIV-1 infected cells or transcytosis of the virus across the epithelium (Maher et al., 2005). Vacharaksa *et al.*

(2008) used tonsil keratinocytes from donors and OKF6/TERT-2 cells to *trans*-infect PBMCs. In fact, the OKF6/TERT-2 cells transferred at least five-fold more HIV-1 than donor tissues as demonstrated by p24 ELISA of the PBMCs (Vacharaksa et al., 2008). Thus, despite there being a low apparent *in vivo* transmission rate (Campo et al., 2006), these studies show that oral and pharyngeal transmission to susceptible cells may occur.

Consistent with evidence of the susceptibility of oral and tonsil epithelium, other *in vivo* data supports the possibility of oral HIV-1 transmission. Not only do oral epithelial cells of HIV-infected patients have HIV-1 DNA integrated into their genomes (Rodriguez-Inigo et al., 2005), but also, non-traumatic exposure of tonsil tissue to SIV and oral exposure to cell-free SIV caused systemic infection in macaques (Baba et al., 1996; Stahl-Hennig et al., 1999). Taken together, these findings demonstrate that epithelial cells of the oral cavity might be productively infected with HIV-1 by cell-borne virus in the absence of CD4 and potentially serve as mediators of systemic infection if they can sequester infectious virions.

In contrast to the oral cavity, the vagina is an accepted susceptible milieu for HIV-1 transmission. Studies with vaginal epithelium have also demonstrated transfer of HIV-1 to susceptible cells by capturing or allowing transmigration of infectious virus. In the late nineties, primary PBMCs from HIV-1 positive patients were shown to transmit virus across many epithelial lines to infect mononuclear cells on the basolateral side of the epithelium (Bomsel, 1997). While human primary urogenital epithelial cells could not be infected with cell-free HIV-1 they were capable of sequestering virus and transmitting it to PBMCs in co-culture (Dezzutti et al., 2001). Carreno *et al.* (2002) demonstrated with HEC-1A cells that R5 HIV-1 transcytosis was enhanced under pro-inflammatory conditions (Carreno et al., 2002). Furthermore, it was shown that an ectocervical genital epithelial cell line sequestered and transmitted HIV-1 to H9 CD4⁺ T cells or PBMC (Wu et al., 2003). Finally, the ability of HIV-1 to retain infectivity after transcytosis across a tight monolayer of primary genital epithelial cells was confirmed by activation of luciferase activity in TZM-bl cells by basally collected medium (Bobardt et al., 2007). However, one study opposes this view of HIV-1 transmission across the mucosa, since recently HIV-1 was found not to cross the vaginal mucosa within 4 h using a model of a reconstituted human epithelium (Bouschbacher et al., 2008). However, epithelium associated sequestered virus may have a longer infectious potential than 4 h (Wu et al., 2003).

1.7.5 Epithelial responses to HIV-1

1.7.5.1 Host cell recognition of pathogens

The innate immune system is the first line of defense against invading microorganisms. Host cell responses to viral, bacterial and fungal microbiota is predominantly mediated by pattern recognition receptors (PRRs), which are well recognised as microbial sensors (Mogensen, 2009). PRRs are triggered by pathogen-associated molecular patterns (PAMPs) expressed by bacteria, viruses and fungi and are comprised of different families. The most recognised include toll-like receptors (TLRs), NOD (nucleotide oligomerization domain)-like receptors (NLRs), the retinoid-inducible gene (RIG)-like receptors (RLRs), C-type lectins (CTLs) and the immunoreceptor tyrosine-based activation (or inhibitory) motifs-containing receptors (ITAM/ITIM) (Mogensen, 2009). The main PRRs associated with viral sensing are the RLRs, predominantly RIG-1 and melanoma differentiation-associated protein 5 (MDA5) in the cytoplasm and TLRs at the cell surface (McCartney and Colonna, 2009) (TLR3, TLR7/8 and TLR9). Viruses are usually recognized by innate cells such as epithelial cells, tissue macrophages, and dendritic cells (DCs) or Langerhans cells that patrol the epithelium through their nucleic acid, dsDNA or ssRNA. Recognition of viruses induces the production of type I interferons, TNF α and other cytokines (Reuter et al., 2012). The production of cytokines signals to neighbouring cells to increase their innate antiviral defences (Reuter et al., 2012). Innate recognition of viruses also allows the activation and instruction of the adaptive immune response: dendritic cells are potent inducers of T cell responses (Carrington et al., 2012). After the virus is captured by DCs, these cells migrate to the local lymph node to process the virus for antigen presentation to T cells, which results in the initiation of Th1 cell proliferation. Once initiated within the lymph nodes, effector Th1 cells travel to the site of infection and eliminate the virus infection when possible (Flint et al. 2009).

1.7.5.2 PRR-mediated signalling and HIV-1

PRR-mediated signalling for bacteria and fungi predominantly involves activation of TLRs and the recruitment of signalling adapters, which ultimately leads to the activation of transcription factors: via the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- κ B), a pathway active in almost all cell types and mitogen-activated protein kinase (MAPK) pathways (p38, JNK (c-Jun N-terminal kinases) and ERK1/2 (extracellular-signal-regulated kinases) and the induction of immune/inflammatory effector (cytokine) responses (Moyes et al., 2010). Single stranded RNA viruses activate especially RIG-1 or TLR7/8 in myeloid cells. They then activate signalling through the adaptor molecule myeloid differentiation primary response gene

(MyD88) via interferon regulatory factor 3 (IRF3), IRF7, NF- κ B and MAPK leading to the production of inflammatory cytokines and Type I interferons (McCartney and Colonna, 2009).

Whilst progress has been made in deciphering viral- and HIV-1-mediated signalling in myeloid cells (Doehle et al., 2009), much less is known in epithelial cells. The majority of epithelial cell work has been bacterial-based and identifies NF- κ B and MAPK as the main pathways activated (Moyes et al., 2010). In addition, our unpublished studies using agonists of TLR3, TLR7/8, showed activation of these aforementioned and IRF3 pathways. Limited data exists regarding HIV-1 activation of epithelial cells, therefore we picked a few candidate pathways to explore. Serious gaps exist in our understanding of HIV-1-epithelial interactions with the immune system and how these might affect establishing a primary infection and /or the progression of HIV-1 disease.

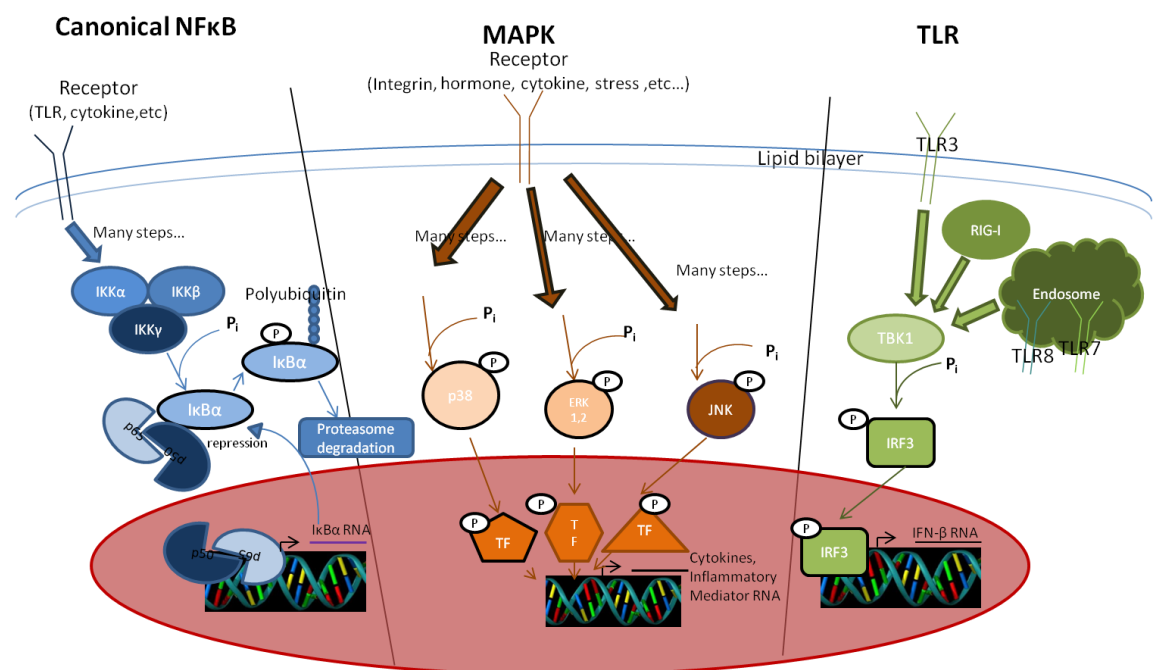


Figure 1-6: Simplified signalling pathways involved in innate immunity with a focus on transcription factors that may have a role to play in epithelial responses to HIV-1. In blue, the intracellular portion of NF- κ B pathway is illustrated. In orange, the three main MAPK pathways are shown and in green, TLR sensors for single stranded RNA viruses are pictured. P represents phosphorylation.

1.7.6 Epithelial responses to HIV-1 and its components

Given that the predominant route of HIV-1 infection is through mucosal surfaces and that HIV-1 has been shown to interact with epithelial tissues, it is surprising how little information is available with regard to how epithelial cells respond to HIV-1. Treatment of epithelial cells with HIV-1 gp120 has been shown to alter cellular responses (Del Corno et al., 2005; Kapasi et al., 2002; Martinelli et al., 2007; Shan et al., 2007; Singhal et al.,

1999). In kidney glomerular epithelium, gp120 increases proliferation at low levels but increases apoptosis and decreases growth at higher concentrations (Singhal et al., 1999). Expression of the transcription factors c-Jun and c-Fos (which are one of many transcription factors that can form the activating protein-1 (AP-1) transcription factor complex), was increased upon treatment with high concentrations of gp120 (Singhal et al., 1999). Following up, apoptosis induced by gp120 in these cells was shown to be dependent on phosphorylation of the MAPK p38 and CD4 expression (Kapasi et al., 2002). Given that the AP-1 transcription factor complex is activated via the MAPK pathway, this implicates the MAPK pathway in epithelial responses to HIV gp120.

It is known that the intestinal epithelium in HIV-1 infected individuals can become dysfunctional allowing bacteria to infiltrate across or invade through the single cell columnar epithelium of the intestine (Epple et al., 2009a; Epple et al., 2009b). Given this, investigators wished to determine whether similar dysfunction was affecting the vaginal epithelium (Nazli et al., 2010). Using electrical transepithelial resistance (TER) and dye, authors showed a decrease in the strength of the tight junctions in epithelial monolayers and decrease in tight junction protein expression (Nazli et al., 2010). Gp120 treatment alone was shown to be sufficient to reduce TER measurement in primary endometrial epithelium and an intestinal epithelial line (Nazli et al., 2010). Finally, the authors showed the cytokines TNF- α , IL-6, IL-10, IL-1 β were significantly increased in apical medium of ADA (R5) HIV-1 exposed primary endometrial epithelium (Nazli et al., 2010). More recently, human mannose receptor (hMR) has been implicated in gp120 binding to vaginal protein lysates and the vaginal epithelial line Vk2/E6E7 since they could be competed out in a dose dependant manner with mannan and by blocking with a hMR antibody (Fanibunda et al., 2011). Gp120 treatment of the cells resulted in significant increase of MMP-9 (Fanibunda et al., 2011), an enzyme that degrades tight junctions, perhaps explaining the mechanism of the relaxation of TER (Nazli et al., 2010). These lines of evidence strongly suggest that the epithelial cells were sensing HIV-1 and were altering their gene expression, morphology and cytokine expression in response to the virus supernatant.

More recently, cyclooxygenase-2 (COX-2) has been shown to be up-regulated in response to Toll-Like receptor (TLR) ligands and TNF- α in human vaginal epithelial cells and ectocervical biopsies (Joseph et al., 2012). This is relevant because COX-2 is an inflammatory enzyme that drives expression of prostaglandin E2, that in turn can drive the expression of the HIV-LTR (Dumais et al., 2002). Therefore, inflammation in the genital tract can lead to changes in gene expression in the genital epithelium, leading to inflammatory signalling from genital epithelium to HIV-1 infected cells, triggering HIV-1 gene expression.

Another publication further characterised a model of heterosexual contact leading to HIV-1 infection. Primary endometrial epithelial cells were shown to respond differentially to HIV-1 infected sperm from men in the acute and chronic phases of infection (Kafka et al., 2012). HIV-1 infected seminal plasma contains more cytokines, chemokines and TGF- β that appear to be different depending on whether the provenance was from an acute phase or a chronic phase patient (Kafka et al., 2012). IL-1 α and TNF- α are increased from endometrial epithelium in response to acute phase seminal plasma while IL-6 is increased in response to uninfected seminal plasma (Kafka et al., 2012). Finally they showed that NF- κ B (positive) and TGF- β (negative) pathways had immunomodulatory roles in responses to uninfected and chronically infected seminal plasma, respectively (Kafka et al., 2012), although the role these pathways play in responses to seminal plasma from acute infected individuals was not addressed.

Another approach to determine epithelial cell responses to HIV-1 is to assess global signalling changes using whole genome expression arrays. However, there is a paucity of microarray data for oral and vaginal epithelial cells. One publication from Acheampong *et al.* (2005) examined primary oral keratinocytes treated with HIV-1 gp120, Nef and HIV-1 Tat and characterised the cells by utilizing a cDNA expression human apoptosis array. These treatments were all found to induce substantial apoptosis at a concentration of 10ng/ml through the TNF/TNF-R Fas/Fas ligand pathways (Acheampong et al., 2005). Although limited to one study, the attainment of such information indicates that comparisons of microarray data from oral and vaginal epithelial cells in response to HIV-1 may identify common signalling pathways and gene profiles important in HIV-1 responses and thus provide a better understanding of HIV-1-epithelial interactions.

Nevertheless, questions remain, including: does HIV-1 activate oral and vaginal epithelial cells and does this promote transmission? Do HIV-1-epithelial interactions lead to cytokine production or a cellular response that would activate or attract immune cells, thus enhancing infection? Mounting evidence suggests that vaginal and cervical epithelium serves more than just an inert structural barrier since their secretions abound with anti-microbial peptides and modulators of innate immunity (Cole et al., 2008).

1.8 HIV-1 and co-infections

In the last few years much research attention has been given to co-pathogens and their effect on HIV-1 infection in the vaginal milieu. Sexually transmitted infections significantly increase the probability of HIV-1 transmission since they not only increase viral shedding in infected individuals, but also increase susceptibility of individuals who

are HIV negative (Galvin and Cohen, 2004; Klotman et al., 2008; Mabey, 2000). This can be due to the epithelial barrier being physically breached by microorganisms, by inflammation and/or by attracting immune cells to the mucosa.

N. gonorrhoeae was shown to induce the expression of human β -defensin-5 and 6, which may recruit susceptible immune cells to the surface (site of infection) where they will be exposed to HIV-1 captured by the vaginal epithelium (Klotman et al., 2008). Endometrial epithelial cells were used to study co-infecting bacteria using TLR ligands or *N. gonorrhoeae* and viral pathogens herpes simplex virus type 1 or 2 (HSV-1/2), and their effect on the expression of the HIV-LTR using a reporter T cell line as a model of HIV-1 genome activation (Ferreira et al., 2011). The genital epithelial cells were briefly exposed to the aforementioned stimuli, extensively washed and their supernatants were collected to study their effect on HIV-LTR activation. Supernatants collected from GECs previously incubated with HSV-1/2, *N. gonorrhoeae*, and TLR ligands gave off apical cytokine signals (IL-6, IL-8, MCP-1, TNF- α) and induced HIV-LTR reporter gene expression (Ferreira et al., 2011). Thus, inflammation brought about by co-pathogens in the vaginal milieu appears to have an effect on HIV-1 by altering gene expression and inflammatory mediators of epithelial cells.

In addition, oral microbiota may promote HIV-1 disease and/or reactivation. The presence of the periodontal pathogen *Porphyromonas gingivalis* correlates with increased CCR5 expression on the surface of oral and tonsil epithelial cells, thereby promoting receptor-mediated recognition and binding of HIV-1 and possible transfer to susceptible immune cells (Giacaman et al., 2008). Also, bacterial extracts from oral pathogens *Fusobacterium nucleatum* and *P. gingivalis* were shown to induce the release of inflammatory signals from an oral epithelial cell line and gingival fibroblasts that could lead to HIV-1 reactivation (Gonzalez et al., 2010). However, bacterial extracts, although perhaps easy to work with, are less physiologically relevant than stimulus by whole bacteria. In addition, *F. nucleatum* and *P. gingivalis* were shown to activate NF- κ B and increase cytokine expression (Milward et al., 2007), therefore potentially having an additive effect in promoting HIV-1 infection in the oral cavity, since NF- κ B has been implicated in response to HIV in other cell types (Giri et al., 2006). Thus, the human microbiota may stimulate inflammation that in turn may promote activation of HIV-1 gene expression and the exposure of susceptible immune cells to the virus in the mucosa. The oral microbiota, e.g. *Candida albicans* may also bind to HIV-1 directly (Gruber et al., 2003) and potentially protect HIV-1 from being cleared by the flow of saliva and mucosal secretions, thereby further facilitating transfer of infectious virus to permissive host cells.

1.8.1 *Candida* and HIV-1 infection

Candida infections occur in approximately 50% of HIV infected and 90% of AIDS patients (Phelan et al., 1987). It is often the first clinical sign presenting in patients that have chronic HIV-1 infection (Flint and American Society for Microbiology., 2009). The close relationship between *Candida* and HIV-1 infection was recently verified in an epidemiological study, which found an association of *Candida* infections with HIV acquisition at two clinics in Zimbabwe and Uganda (van de Wijgert et al., 2008).

Human mucosal surfaces normally have a commensal relationship with *C.albicans* but this depends on the maintenance of a varied population of normal microbial flora and the maintenance of tissue integrity by a healthy immune system (Henriques et al., 2006). The progression of *Candida* infection involves the perturbation of the mucosal microenvironment to allow adherence of the yeast form, leading to hyphal formation and eventually hyphal elongation to invade the mucosal barrier, in severely immunocompromised individuals this is followed by growth and dissemination through the blood system to cause a systemic infection (Henriques, et al 2006).

Many factors govern *Candida* virulence. Firstly, three families of hydrolases exist: secreted aspartyl proteinases (Sap), secreted lipases (Lip), phospholipase B (Plb). These contribute to the provision of nutrients and dissolve tissues to promote fungal penetration of host barriers (Brown et al., 2007; Henriques et al., 2006; Schaller et al., 2005). In addition, adhesins such as the Als family and Hwp1, both responsible for attachment to epithelial cells and promoting hyphal morphogenesis, contribute to *Candida*'s interface with the host (Brown et al., 2007; Hoyer, 2001).

Like other mucosal pathogens, *C. albicans* may also activate epithelial responses to induce inflammation, which may subsequently affect HIV-1 progression. Recently, our group identified that in oral epithelium *C. albicans* triggers an initial NF- κ B and MAPK response based on recognition of common polysaccharides present in the cell (chitin, β -glucan, mannan), followed by a second, stronger MAPK activation event that is dependent on fungal burdens and the formation of hyphae (Moyes et al., 2010). In vaginal epithelium, the initial MAPK activation is slightly delayed but the second phase is common to both epithelial sites (Moyes et al., 2011). These are similar pathways to those implicated in HIV-1 infection in immune cells (Giri et al., 2006). In addition, *C. albicans* induces the expression of COX-2 in HeLa cells via TLRs (Deva et al., 2003), which is an enzyme that processes prostaglandins and leads to mucosal inflammation (Joseph et al., 2012). Thus, *C. albicans* specifically increases inflammation and affects signalling pathways and loss of epithelial barrier function in similar ways to HIV-1. Therefore, their effects may be additive and promote HIV-1 infection. This would correlate with epidemiological data, which showed that women with vaginal *Candida*

infections were more likely to acquire HIV-1 especially if the *Candida* infection was present at the time of HIV-1 detection or at the previous visit (van de Wijkert et al., 2008). However, the authors failed to find a relationship between disrupted vaginal flora and immune activation but acknowledge that monitoring pro-inflammatory cytokines may have been more rigorous than the cytology they performed (van de Wijkert et al., 2008).

Most of the studies on *Candida* and its direct interaction with HIV-1 originate from the Wurzner group from 1997 to the present. The binding of the HIV-1 envelope to *C. albicans* using two competition assays suggested that gp41 was involved in this binding event (Wurzner et al., 1997). The following study showed that HIV-1 envelope (gp160) bound to the fungal surface and increased *Candida* adhesion to HIV-1 infected cells. gp160 binding also decreased phagocytosis of *Candida* and increased the production of the Sap from *Candida* (Gruber et al., 1998). The authors followed this by claiming that HIV-1 Tat was able to induce hyphal growth (Gruber et al., 2001). Subsequently, HIV-1 gp41 was shown to bind to different *Candida* species (*C. albicans*, *C. dubliniensis* and *C. tropicalis*). Glycoprotein gp41 was able to modulate *C. albicans* adhesion to HIV-1 infected T cells as well as uninfected epithelial and endothelial cells (Gruber et al., 2003). Most recently, the group claimed to have identified an HIV-1 interacting protein in *C. albicans*: Hgt1p (Lesiak-Markowicz et al., 2011). However, the evidence provided for Hgt1p-HIV-1 binding is tenuous, since binding to whole virus particles was not verified. Instead, the authors demonstrated gp160 binding was reduced by ~30% to a $\Delta hgt1$ null mutant. However, the $\Delta hgt1$ mutant was not able to form hyphae like the parent and revertant strains (Lesiak-Markowicz et al., 2011), so if the yeast form of *C. albicans* binds the gp41 portion of gp160 less avidly than hyphae (Gruber et al., 2003), then this could account for the reduction in binding to gp160.

Work on another human RNA retrovirus, human T-lymphotrophic virus (HTLV), has shown that this virus forms a biofilm: a carbohydrate covered, glycosylated “cocoon”. This disguise, both enhances infectious capacity by allowing the virus to bind host cells, improves transmission between cells within the host and increases immune evasion (Pais-Correia et al., 2010; Thoulouze and Alcover, 2011). This virus shares similarities with HIV-1 in that it is composed of two single-stranded RNA genomes and that it originated in primates. Although work with HIV-1 in this field has not yet been published to our knowledge, there is now a significant level of interest in this area as evidenced by presentations on this topic at the HIV symposium held at the Barts and the London (July 4th 2010). Thus, it is possible that given the highly glycosylated nature of gp120, HIV-1 may adhere to biofilms laid down on mucosal surfaces by the host microbiota, including the carbohydrate/polysaccharide moieties of *Candida* species. HIV-1 may form a biofilm of its own since 50% of the molecular mass of HIV-1 gp120 is constituted of N-linked

glycans (Allan et al., 1985; Checkley et al., 2011) with a small addition of O-linked glycans (Bernstein et al., 1994). Notably, gp120 harbours 20-35 N-linked glycosylation sites and gp41 harbours 3-5 N-linked glycosylation sites (Checkley et al., 2011). *Candida* also has an abundance of glycosylation sites for attachment of N-linked and O-linked glycans. Mutants defective in N- and O- glycosylation have been developed to assess yeast cell-cell interactions (Bates et al., 2006; Bates et al., 2005; Munro et al., 2005). Therefore, it is possible that HIV-1 may interact with *C. albicans* via these glycosylation moieties.

1.9 Project Aims

Given the ambiguous literature on the interactions of HIV-1 with oral and vaginal epithelial cells and *Candida*, this project aimed to determine whether HIV-1 binds, enters and integrates into oral epithelial cells, whether productive infection ensues, and whether sequestered infectious virus can be transferred to permissive cells. We also investigated which epithelial signalling pathways HIV-1 activates and how HIV-1 modulates epithelial cells on a global scale. Finally, we investigated the ability of HIV-1 and *Candida* to directly interact with each other, and whether this mucosal fungal pathogen was able to sequester and transfer infectious virus to permissive cells. To undertake these investigations, we utilized epithelial cell lines representative of oral and vaginal sites that are rarely investigated with regard to epithelial HIV-1 transmission but are one of the first cell types that are likely to come into contact with HIV-1. These were buccal (TR146), oro-pharyngeal (FaDu) and vulvovaginal (A431) epithelial cells, all of stratified squamous cell origin.

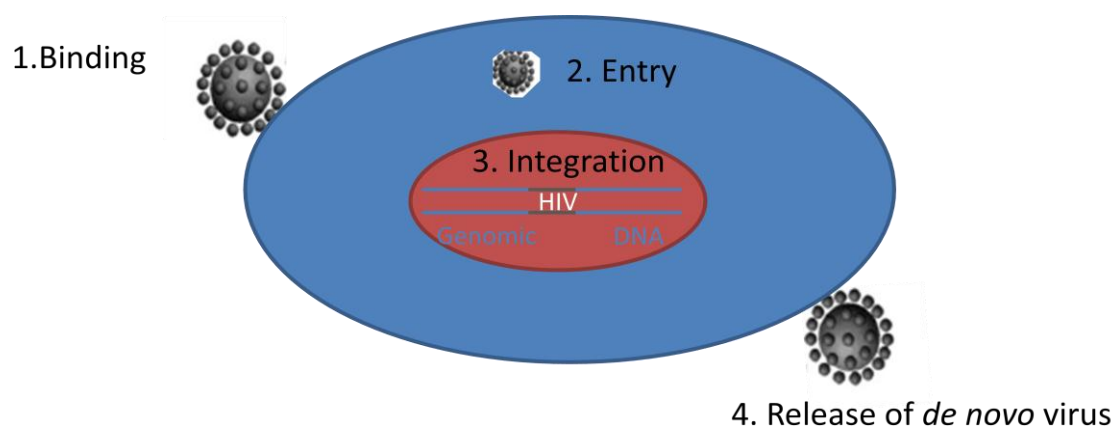


Figure 1-7: Simplified model of HIV life cycle to be tested in this thesis work. We wished to assay binding, integration of HIV-1 and infectious virus release. HIV-1 particle taken from (Sierra et al., 2005).

The specific project aims, outlined in Figure 1.7, were:

1. To characterise epithelial models of oral, oro-pharyngeal and vaginal epithelial cells for RNA and protein expression of HIV-1 receptors, co-receptors and binding factors.
2. To determine whether HIV-1 binds to epithelial cells.
3. To ascertain whether integration (as a surrogate for infection) occurs upon HIV-1 exposure to epithelial cells.
4. To assess whether HIV-1 is transferred from epithelial cells to susceptible reporter cells.
5. To determine which cytokine, intracellular signalling or global gene expression changes occurred in epithelial cells in response to HIV-1 exposure.
6. To characterise the interaction events that occur between HIV-1 and *Candida* and whether *C. albicans* is able to transfer virus to susceptible cells.

Chapter 2 Materials and Methods

2.1 Cell lines, culture and reagents

Human oral buccal (TR146), oro-pharyngeal (FaDu), and vulvo-vaginal (A431) epithelial carcinoma cell lines and renal epithelial (293T) cells were obtained from the American Type Culture Collection (ATCC).

TR146 cells are a human buccal carcinoma cell line originally isolated from a neck metastasis (Rupniak et al., 1985). These cells form undifferentiated, non-keratinized stratified epithelium that share many functional and morphological characteristics of normal buccal mucosa. FaDu cells are a pharyngeal cell carcinoma line (Rangan, 1972). A431 (also designated A-431) cells are human epidermoid carcinoma of vulval origin (Giard et al., 1973). All three cell lines are commonly used in infection studies and we have utilised the TR146 and A431 cell line extensively in our previous studies (Moyes et al., 2011; Moyes et al., 2012; Moyes et al., 2010; Murciano et al., 2011).

The following reagents were obtained through the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH: TZM-bl cells (catalogue no. 8129), PM1 cells (no. 3038), C8166 (no. 404) and JLTRG-R5 (no. 11586).

TZM-bl are a HeLa derived reporter cell line used for HIV-1 infection studies (Derdeyn et al., 2000). HeLa cells originated from what was diagnosed as an epidermoid carcinoma (Scherer et al., 1953) but were later thought to be adenocarcinoma of the cervix (Jones et al., 1971). The clone originally named JC53-BL expresses CXCR4 and was engineered to express CD4 and CCR5 (Platt et al., 1998). The cells were further modified to incorporate reporter cassettes expressing luciferase and β -galactosidase under the control of an HIV-1 long terminal repeat (LTR: the retroviral promoter sequence) where expression of the reporter is triggered by the production of HIV-1 *tat* (Derdeyn et al., 2000).

Three cell lines were used for virus propagation. C8166 are human umbilical cord blood T lymphocytes (Salahuddin et al., 1983). NP2-R5 cells are human glioma cells expressing CD4 and CCR5 (Soda et al., 1999). JLTRG-R5 cells are a Jurkat T cell-based reporter cell line expressing CD4 and both HIV-1 co-receptors, CXCR4 and CCR5, and are derived as a spontaneously occurring sub-clone of JLTRG cells (Ochsenbauer-Jambor et al., 2006).

TR146, FaDu, A431, TZM-bl and 293T are all adherent cells maintained in Dulbecco's Modified Eagle Medium (DMEM, 4.5 g/l glucose and L-glutamine) (PAA laboratories) supplemented with 10% foetal calf serum (FCS) and 1%

Penicillin/Streptomycin (P/S) (DMEM 10). They were passaged every 2-3 days. TR146, 293T and FaDu grew rapidly and were split 1:6 to 1:8. On the other hand, A431 and TZM-bl cells grew at a slower rate and were split 1:3 or 1:4.

NP2-R5 cells were additionally supplemented with 1 mg/mL of G418 (Invitrogen) and 1 µg/mL of puromycin (Invitrogen) in DMEM supplemented with 10% FCS and 1% P/S. They were passaged every 3-4 days at split 1:4.

C8166, JLTRG-R5 and PM-1 cell are suspension cells and were maintained in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FCS and 1% P/S. They were passaged every 3-4 days at split 1:10.

2.2 Plasmid isolation

Plasmids were used in transfections to generate infectious viruses (see section 2.3.1) Plasmids were transformed into *Escherichia coli* DH5α competent cells. Typically 100-200 ng of plasmid DNA was incubated with freshly thawed cells for 25-30 min, incubated for 30 s at 42°C, then fed with 950 µg Lysogeny broth (LB) (10 g/L Tryptone, 5 g/L yeast extract, 10 g NaCl/L in H₂O and immediately autoclaved). The tube was then incubated with shaking at 37°C for 1 h and then streaked onto an LB agar (same as LB with 15 g/L agar added) plate with appropriate selection antibody (typically Ampicillin: 50 mg-75 mg/L). The plate was then incubated at 16 h in 37°C.

For HIV molecular clones, an *E. coli* DH5α colony was selected from the plate, inoculated to LB ampicillin medium and grown for 8 h with shaking at 37°C. Then the liquid medium was spread and dried to an LB ampicillin agar plate and incubated at 37°C and the *E. coli* DH5α cells grown to confluence. Cells were scraped using a sterile loop and the DNA isolated by NucleoSpin® Plasmid miniprep kit (Macherey-Nagel). This would typically yield about several µg of DNA.

If more DNA was required the *E. coli* DH5α colony from the plate was grown in 250 mL LB ampicillin (50 mg-75 mg/L) liquid medium and incubated with shaking for 16 h. The DNA was harvested the following day using the EndoFree Plasmid Maxi Kit (Qiagen).

2.3 Virus growth

2.3.1 Virus growth by transfection

The following reagents were obtained through the ARRRP: HIV-1 molecular clones pYU2 (R5-utilizing, catalogue no.1350) and pLAI.2 (X4-utilizing, catalogue no. 2532), HIV-gpt (gp160 deficient HIV molecular clone catalogue no. 1067), pYK-JRCSF (R5-utilizing, catalogue no. 2708), pNL4.3 (or NL4-3, an X4-utilizing, catalogue no. 114).

Briefly, 293T cells were seeded at 95% confluency in a 10 cm³ dish. The following day cells were transfected with 5 µg of the infectious molecular clone pLAI.2 (X4) or pYU2 (R5) or pJRCSF (R5) or 10 µg HIV-gpt used per transfection using the polyanionic transfection reagent Jet PEI (10 µL JetPEI per 5 µg DNA with 250 µL of included NaCl solution) (Polyplus Transfection). After 24 h, the media was replaced and at 48, 72 and 96 h post-transfection virion-containing culture supernatants were harvested and filtrated through a 0.45 µm pore size membrane and stored in aliquots at -80°C until required for plaque assay (section 2.3.3).

2.3.2 Virus growth by passaging through susceptible cells

High titer virus stock (initially made by the above transfection protocol) in DMEM 10 was thawed, and about 1 ml was added to 1-2x10⁶ cells (see below) in small volume for 1-3 h then transferred to a T75 flask. For YU2-R5 virus, the infection was started with NP2-R5 cells and for LAI-X4 virus, C8166 suspension cells were used. For control conditions, cultures were started with the same number of NP2-R5 or C8166 cells but without the addition of virus. Once cytopathic effects (CPE) were visible, more cells were added (JLTRG-R5 for YU2 or C8166 for LAI) to the virus and control conditioned medium stocks, and the stocks were monitored daily for acidification of the medium and proportion of the cells displaying CPE in the virus stock. The stocks were fed with medium (DMEM 10) until no more CPE was observed accumulating (usually 1-2 weeks). The virus stocks were frozen in equal aliquots with equal amounts of cells. When required, a small aliquot was thawed, clarified by centrifugation at 15000rpm for 10 min assayed by plaque assay (section 2.3.3). Aliquots with an approximated titer were thawed, clarified and used as required. On use, virus stocks were retitrated. This method yielded ten-fold higher titer (approximately 1x10⁵ to 1x10⁶ infectious units per mL) viruses as compared to transfection and was more cost effective.

2.3.3 Virus quantification

Infectious virus stock (LAI-X4, YU2-R5, JRCSF and NL4.3) titers were determined by plaque assay. Briefly, TZM-bl cells (1 × 10⁴ cells/well) were cultured overnight (96-well plates) and incubated with eight replicates of ten serial dilutions (0.5 log) of virus stock in a total of 50 µL DMEM 10 per well. After 16 h, 50 µL of DMEM 10 was added to each well. After 48 h, virus supernatant was removed and the cells were fixed with 0.05% glutaraldehyde for 10 min at room temperature and washed twice with phosphate-buffered saline (PBS). Expression of β-galactosidase was determined by staining cells with X-Gal stain [1 mg/mL X-Gal in 5 mM KFe₄(CN)₆ 3H₂O, 5 mM KFe₃(CN)₆ 3H₂O, and 1 mM MgCl₂] and incubating culture plates at 37°C for 1 h. Virus infectivity was estimated as plaque forming units (PFU) per mL.

Since this plaque assay was not effective for quantification of HIV-gpt, p24 ELISA (ZeptoMetrix Corporation, Franklin, MA, USA) was used according to the manufacturer's instructions (by Carlo Scala alongside his samples) to estimate concentration and extrapolated to multiplicity of infection (MOI) using a few viruses whose MOI was known.

2.4 HIV-1 receptor expression

2.4.1 HIV-1 receptor surface protein expression by flow cytometry

TZM-bl, NP2-X4, NP2-R5, TR146 and FaDu resting cells were washed with PBS and incubated with 0.02% (W/V) EDTA for 5-30 min. Detached cells were washed thoroughly with PBS supplemented with 1% BSA and 0.01% azide (wash buffer), and resuspended at 1×10^6 cells in 1 mL wash buffer. To identify surface expressed HIV-1 receptors and co-receptors, 100 μ L of cells were incubated at room temperature for 1 h with mouse anti-human CD4 (1:4 catalogue no.724), CCR5 (1:20, catalogue no. 4090), CXCR4 (1:80, catalogue no. 4083), monoclonal antibodies (all obtained from the ARRRP), DC-SIGN (1:100, Biolegend), GalCer (1:200, anti-galactocerebroside, Millipore) or heparan sulfate proteoglycan (1:200, Millipore), and ICAM-1 (a kind gift from Annapurna Vyakarnam) monoclonal antibodies. Primary antibodies were detected with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch) except the ICAM-1 which was directly conjugated with phycoerythrin (PE). After thorough washing, cells were fixed in 200 μ L 4% formaldehyde and the percentage of FITC-expressing cells was determined by flow cytometry.

2.4.2 HIV-1 receptor gene expression by real-time PCR

RNA was isolated from resting TZM-bl, TR146, FaDu and A431 cells using GenElute Mammalian Total RNA Miniprep Kit (Sigma) or Nucleospin II kit (Macherey Nagel), followed by treatment with Turbo DNase free (Ambion) according to the manufacturer's instructions. All samples were confirmed DNA free prior to analysis. cDNA was synthesized from 1 μ g of RNA using HIV Reverse Transcriptase (Ambion) according to the manufacturer's instructions. Primers were obtained from RTPPrimerDB (<http://medgen.ugent.be/rtprimerdb/>) (Lefever et al 2008) and PrimerBank (<http://pga.mgh.harvard.edu/primerbank>) (Spandidos, 2010). Gene expression of CD4, CCR5, CXCR4, DC-SIGN, SDC-1 (syndecan-1) and SDC-4 (syndecan-4) was calculated by quantitative real-time PCR using SYBR Green JumpStart Taq Ready Mix (Sigma) with 4 pmol primers and 1 μ L cDNA in 10 μ L reactions on the Corbett Research Rotor-Gene 6000 (Qiagen) using the following cycling parameters: 95°C for 3 min; followed by 95°C for 3 s, annealing for 10 s and extension for 20-30 s for 40 cycles at the temperatures listed in Table 2-2. Data was analysed with Corbett Research Rotor-Gene

Table 2-1: Primer sets utilized to assess HIV-1 related receptor gene expression

Host target	Entrez no.	Primer sequence 5'-3'	Primer location (exon)	Annealing temperature	Amplicon size	Relationship between gene and HIV-1	R ²
CD4	920	F:ACTAAAGGTCCATCCAAGCTGA R:GCAGTCAATCCGAACACTAGCA	3,4	60	151	HIV receptor (Klatzmann et al., 1986)	0.997
CXCR4	7852	F:CCTCATCCTGGCTTTCTTCG R: GAATGTCCACCTCGCTTTCC	1	60	285	HIV co-receptor T-cell trophic strains, formerly "fusin"(Feng et al., 1996)	0.953
CCR5	1234	F: TGGACCAAGCTATGCAGGTG R: CGTGTCAACAAGCCCACAGAT	3	58	240	HIV co-receptor macrophage trophic strains (Alkhatib et al., 1996)	0.992
DC-SIGN (CD209)	30835	F:TCAAGCAGTATTGGAACAGAGGA R:CAGGAGGCTGCGGACTTTTT	7	60	136	Dendritic cell specific HIV-1 binding protein that enhances trans infection of T cells (Geijtenbeek et al., 2000)	0.995
SDC-1	6382	F:TGAAACCTCGGGGGAGAATAC R:GGTACAGCATGAAACCCACC	3,4,5	60	171	Required for HIV-1 tat protein accumulation in endothelium (Urbini et al., 2009)	0.992
SDC-4	6385	F:CAGGGTCTGGGAGCCAAGT R:GCACAGTGCTGGACATTGACA	4	58	129	Complexes with CXCR4 (Hamon et al., 2004)	0.985
ICAM-1	3383	F:TGCCCCGAGCTCAAGTGTCTA R: CCCTTTTTGGGCCTGTTGTA	6,7	58	264	Increases viral binding, promotes infectivity (Paquette et al., 1998)	0.984
β-actin	60	F:CATGTACGTTGCTATCCAGGC R:CTCCTTAATGTCACGCACGAT	4	58	250	Reference gene	0.996

Table 2-2: qPCR cycling conditions

Primer	Initial Denaturation		Cycling times (40 cycles)		
CCR5, β-actin	95°C 3min	95°C 3s	58°C 10s	75°C 30s	(Read at 75°C)
SDC1, CD4	95°C 3min	95°C 3s	60°C 10s	75°C 20s	(Read at 75°C)
SDC4, ICAM-1	95°C 3min	95°C 3s	58°C 10s	72°C 20s	Read at 80°C 5s
CXCR4, DC-SIGN	95°C 3min	95°C 3s	60°C 10s	75°C 30s	Read at 80°C 5s

6000 Series Software 1.7 using the two standard curve method with β -actin used as the normalizer gene.

2.4.2.1 HIV-1 receptor expression by real-time PCR on *C. albicans* exposed epithelial lines

1×10^6 cells were seeded to 6-well tissue culture plates and inoculated with 2×10^4 yeast cells in 1 ml volume DMEM 1% P/S. After 24 h the cells were washed, and RNA isolated as detailed in the previous section.

2.4.3 Flow cytometry for HIV gp120 detection at the surface of epithelial cells

Prior to the method being definitely established, many antibodies from the ARRRP were screened to determine if they could be used to monitor the presence of gp120 envelope of whole virus or p24 on the cell surface. Antibodies against gp120 assayed included catalogue numbers: 810, 2640, 7369, 2343, 1476, 7371 (see Appendix C for their properties) as well as those we ultimately used: 7626 (F425 B4e8) and 7624 (F425 A1g8). Antibodies against p24 assayed for flow cytometry included catalogue numbers: 6521, 1238, 530, 4121, 6457. None were used in flow cytometry. Those selected were chosen because the presence of HIV-1 could be detected by flow cytometry (by an increase in percent fluorescence) on epithelial cells. Once it was established that HIV-1 gp120 could be detected on the surface by flow cytometry, the blocking and washing conditions were then optimized.

ARRRP # Name	Isotype	Epitope	Neutralizes primary isolates	References
7624 F425 A1g8	IgG ₁ ,K	CD4i epitope of gp120; binding enhanced by sCD4	yes	Pantophlet et al., 2007, Bell et al., 2008
7626 F425 B4e8	IgG ₂ ,K	Base of V3 loop of gp120	yes	Pantophlet et al., 2007, Bell et al., 2008

5×10^4 target cells were infected with 5 infectious units per cell (MOI of 5) of either YU2-R5 virus or LAI-X4 virus. The cells were incubated for 18 h at 4°C, then washed with PBS three times before being blocked in PBS 10% BSA for 10 min at room temperature. Cells were then scraped from the plate and stained for 30 min at 4°C with NIH HIV-1 gp120 monoclonal antibody (F425 B4e8) or a second monoclonal where indicated (F425 A1g8) at a 1/200 dilution in PBS/10% BSA. These monoclonals were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and were made available by Dr. Marshall Posner and Dr. Lisa Cavacini.

After labelling, the cells were washed three times with PBS and then stained with CyTM5-conjugated AffinityPure Goat Anti-Human IgG secondary antibody (Jackson

ImmunoResearch Laboratories, INC.) at a dilution of 1/400 in PBS/10% BSA and incubated at 4°C for 30 min. The cells were washed three times with PBS, resuspended in 4% formaldehyde, and subjected to flow cytometry analysis on FACSCanto machine using the FACSDiva software. The FCS file data was then analysed using WinMDI. CyTM5 fluorescence was read on APC channel. Percentages were calculated as increased in CyTM5 shift from the scatter plot of uninfected primary and secondary stained cells.

2.4.4 Trypsin sensitivity and flow cytometry

TZM-bl, TR146 and FaDu cells (5×10^4) were infected with YU2-R5 or LAI-X4 virus at an MOI of 5, or no virus overnight at 4°C. Cells were washed three times with PBS and then trypsinised for 5 min at 37°C (control cells were left without trypsin). Trypsin was inactivated with DMEM 10 and washed with PBS. All cells were blocked in PBS/10% BSA for 10 min at room temperature. Cells were gently removed by scraping, centrifuged and labelled with NIH HIV-1 gp120 monoclonal antibody F425 B4e8 (1:200) and then Cy5-conjugated AffinityPure goat anti-human IgG secondary (1:400) (Jackson ImmunoResearch) in blocking buffer, each for 30 min at 4°C. Cells were washed three times with PBS, resuspended in 4% formaldehyde and subjected to flow cytometry. Binding percentages were calculated as increased Cy5 shift of the infected and stained cells from infected secondary alone stained cells.

2.5 Western Blotting

2.5.1 Detection of p24 and signalling proteins

TR146 and A431 cells were seeded at 5×10^5 cells per well, grown overnight, and then serum starved using serum free DMEM with 1% P/S for 24 h. Cells were then infected with YU2-R5 or LAI-X4 virus at an MOI of 5. After 2 h incubation at 37°C (or 24 h for p24 binding Westerns) the cells were washed three times to remove unattached virus. Cells were harvested in 150 μ L 1 x RIPA buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS, supplemented with Halt complete protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitors (Perbio Science), placed on ice for 30 min, scraped, clarified by centrifugation for 10 min at 14,000rpm, and stored at -80°C until required. Total protein lysates (mammalian) were normalized for protein content using the bicinchoninic acid (BCA) assay (Pierce) and separated using 12% SDS-PAGE gels. 10-20 μ g of total protein was loaded per sample per well. 10% Dithiothreitol (DTT) and Thermo Scientific Pierce Sample Loading Buffer (4X), a non-reducing lithium dodecyl sulfate sample loading buffer (LDS), were added and the sample heated at 80°C for 10 min prior to loading. After gel electrophoresis (usually 120 V for 1 h), proteins were transferred to PVDF membranes (100V for 90 min)

and probed with the monoclonal antibodies listed in Table 2-3 to detect activation (phosphorylation) of epithelial signalling proteins. Primary antibodies were detected with the appropriate secondary IgG horseradish peroxidase (HRP)-conjugated antibody (Jackson ImmunoResearch) before developing using Immobilon-ECL (Millipore). α -actin was used as a loading control and p24 to detect presence of virus. The Western blot method was optimized in the laboratory by Arinder Kohli (p24) and David Moyes (other antibodies) (Moyes et al., 2010).

Table 2-3: Detection antibodies for epithelial signalling proteins

Antibody 1:1000	Band sizes (kD)	Secondary 1:10000	Specificity	Catalogue number, company
p24	55 for p55, 38 for p38, 24 for p24	Anti-Mouse	Epitope not mapped	6457, ARRRP (Simon et al., 1997)
Phospho SAPK/JNK	57 phospho p54, 46.5 phospho p46	Anti-Rabbit	Phosphorylation at Threonine 183 and tyrosine 185	9251, Cell Signalling Technology
Phospho-p38 MAPK	40	Anti-Rabbit	Thr 180/Tyr182	4511, Cell Signalling Technology
Phospho-p44/42 MAPK (Erk1/2)	42, 44	Anti-Rabbit	Thr202/204	4370, Cell Signalling Technology
Phospho-I κ B- α	30-40	Anti-Rabbit	Ser 32	2859, Cell Signalling Technology
Phospho-IRF-3	55	Anti-Rabbit	Ser 396	4947, Cell Signalling Technology
α -actin	42	Anti-Mouse	N-terminal 2/3 possibly near 50-70	1501, Chemicon MAB

2.5.2 Detection of HIV-1 p24 on fungal species.

The fungal species used in this study and fungal growth conditions are listed in section 2.11 below. 1×10^5 fungal cells from a culture grown overnight with shaking at 30°C were aliquoted into a 1.5 mL tube and resuspended with DMEM 10 and 1% P/S with or without virus as indicated (either YU2-R5, LAI-X4, JRCSF-R5 or NL4.3-X4 at MOI of approximately 1, confirmed by retitration alongside experimental samples in 1 mL total volume). Cells were incubated at 37°C with a gently shaking platform for 2 h or 24 h as indicated. The fungal cells were washed three times with cold PBS and the fungal cell pellets resuspended in 10 μ L Laemmli's loading dye containing DTT. The samples were then boiled for 10 min at 80°C, 15 μ L per well were loaded and separated using 12% SDS-PAGE gels. Proteins were transferred to PVDF membranes, probed with anti-HIV-1 gag monoclonal antibody recognizing p24 and p55 isoforms (ARRRP catalogue no.

6457) and a secondary anti-mouse IgG horseradish peroxidase (HRP)-conjugated antibody (Jackson ImmunoResearch), before developing, using Immobilon-ECL (Millipore).

2.6 Cell damage assay

Epithelial cell damage was determined at 24 h by measuring lactate dehydrogenase (LDH) activity in the culture supernatant as described previously (Moyes et al., 2010; Murciano et al., 2011). This was performed using the Cytotoxicity 96 nonradioactive cytotoxicity assay kit (Promega) according to the manufacturer's protocol and using a recombinant porcine LDH (Sigma-Aldrich) to generate a standard curve. Sample values were then extrapolated from this standard curve (Murciano et al., 2011).

2.7 Integrated HIV-1 PCR Assays

2.7.1 Alu-LTR two-step PCR

To determine whether HIV-1 DNA was able to integrate into epithelial cells, a nested PCR was performed initially with HIV-1 and human Alu-specific primers as previously described (Brussel and Sonigo, 2003, 2004). TR146, FaDu and PM-1 (control) cells were seeded at 5×10^5 cells per well and the following day infected with YU2 (R5) or LAI (X4) virus at an MOI of 0.2. After overnight incubation at 37°C, unbound virus was removed by washing and total DNA was isolated using the GeneElute DNA Extraction kit (Sigma) according to manufacturer's instructions. A 2 µL aliquot of DNA was used in first round PCR to amplify Alu-LTR sequences using 10 pmol forward primer L-M667 (5'- ATG CCA CGT AAG CGA AAC TCT GGC TAA CTA GGG AAC CCA CTG-3') and 30 pmol of Alu-1 (5'- TCC CAG CTA CTG GGG AGG CTG AGG-3') and Alu-2 (5'- GCC TCC CAA AGT GCT GGG ATT ACA G-3') reverse primers in a 20 µL total volume containing 1 x PCR buffer, 100 µM dNTP's, 1.5 mM MgSO₄, 2.5 U *Taq* polymerase (New England Biolabs). Cycle parameters were as follows: 95°C for 8 min; followed by 95°C for 10 s, 60°C for 10 s, 72°C for 170 s for 12 cycles. A 1 µL aliquot of the first round product was used in the subsequent nested PCR, comprising 30 pmol of forward primer λT (5'- ATG CCA CGT AAG CGA AAC T -3') and reverse primer AA55M (5'- GCT AGA GAT TTT CCA CAC TGA CTA A-3'), in a 10 µL reaction containing 1 x SYBR Green JumpStart *Taq* Ready Mix (Sigma). Cycle conditions were as follows: 95°C for 8 min; followed by 95°C for 10 s, 60°C for 10 s, 72°C for 9 s for 40 cycles. PCR products were resolved on 2% agarose gels stained with ethidium bromide. This method

was optimized by Arinder Kohli, and was further tested and proven to be prone to false positives in Chapter 5 (5.3.1).

Where indicated two drugs from ARRRP were used to inhibit integration by pre-treatment for 4 h at 37°C prior to incubation with infectious HIV-1: AZT (no 3485) 500 µM diluted in 10% FCS, 1% P/S medium and Integrase Inhibitor (118-D-24 or 4-[3-(azidomethyl)phenyl]-2-hydroxy-4-oxo-2-butenic acid, no 9957) 25 µM in SFM.

2.7.2 Primer-Probe PCR assay

The primer-probe assay was based on the protocol described by Mbisa et al (2009). 1×10^5 TR146, A431, FaDu and either C8166 or NP2-R5 were plated and infected or incubated the following day with indicated viral MOI (1-144). Virus stocks were pre-treated with RNase-free DNase (Roche, Welwyn Garden City, UK) at 37°C for 1 h in 1x buffer, with an equivalent volume of untreated (not DNase treated) controls. The virus stock that was left untreated was then heat inactivated 60-65°C for 1 h. This heat inactivated virus is unable to infect cells and acts as a control giving a measure of the level of DNA contamination in the system. Cells were incubated with DNase-treated and heat inactivated virus stocks at the stated MOI for 48 h with addition of medium if the medium was acidifying. The cells were washed three times with PBS and DNA harvested with GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, Poole, UK) according to the manufacturer's instructions except the samples were treated with Proteinase K for longer (20 min). DNA samples were digested with DpnI enzyme (New England Biolabs, Hitchin, UK) (1 unit to 1x buffer 4) to degrade any plasmid DNA contaminant (the enzyme has a short recognition site and cuts to produce blunt fragments and is blocked by CpG methylation in mammalian DNA but not by dam methylation in *E. coli*) for 2 h with 20 min heat inactivation at 80°C. DNA was then quantified by Nanodrop and 50 ng or 100 ng of cellular DNA was used in real time PCR (all samples from the same cell line were compared at same DNA concentration) using primers 0.2 M MH535 forward (5'-AACTAGGGAACCCACTGCTTAAG-3') and 0.8 M reverse SB704 (5'-TGCTGGGATTACAGGCGTGAG-3') with 0.2 M probe P-HUS-SS1 (5'-FAM-TAGTGTGTGCCCCGTCTGTTGTGTGAC-TAMRA-3') (Mbisa *et al.* 2009) with Sigma JumpStart™ Taq ReadyMix™ (Poole, UK) in 10 µL reactions. Samples were denatured for 10 min followed by cycling conditions 94 °C for 15 s, 60°C for 30 s and 72°C for 60 s for 60 cycles of amplification in a Corbett Rotor-Gene machine. DNase treated virus exposed samples were compared with heat inactivated virus-exposed samples amplified from an equal concentration of starting template. A positive integration event is demonstrated when a lower C_t value is observed in the DNase treated virus sample

when compared with the heat inactivated virus. A negative integration event occurred when the DNase treated virus-exposed sample showed either no amplification or a higher C_t value than the heat inactivated virus-exposed sample. This method was used to verify whether epithelial integration of HIV occurred in section 5.3.2.

2.8 Virus transfer from epithelial cells to TZM-bl

FaDu, TR146, A431 and TZM-bl cells were seeded at 1×10^5 cells and the following day infected with YU2 (R5) or LAI (X4) virus at an MOI of 0.2. After 16-18h incubation at 37°C the cells were thoroughly washed three times (PBS) to remove any unbound virus. Controls included incubation of cell lines without the addition of virus. TZM-bl cells (3×10^5) were then overlaid onto the epithelial cells and the plates incubated for a further 48 h at 37°C. Cells were fixed and stained for β -galactosidase expression with X-Gal stain. Individual wells were photographed by light microscopy at 200 X magnification.

2.9 Cytokine induction by gp120/140 by Luminex assay

TR146 and A431 cells were grown to confluence and then serum starved in serum-free medium (SFM) with 1% P/S for 2 h prior to stimulation with HIV-1 envelope recombinant glycoproteins gp120 BAL-R5 that binds CD4 (no 4961, expression construct by Dr. Marvin Reitz), recombinant full-length glycosylated CN54-X4 Clade C produced in insect cells using the baculovirus expression system. The protein is produced in as a fusion with maltose binding protein. It also contains a C-terminal His tag. (no 7749, Morikawa et al., 1990) (both from ARRRP) and mammalian glycosylated oligomeric gp140s UG21-X4 (Jefferis et al., 2004, 2006) and ZM96 Clade C (Cat no ARP696 from Programme EVA Centre for AIDS Reagents, provided by Polymun GmbH) all at a concentration of 1 μ g/mL in SFM. No cellular toxicity was obvious from microscopic examination. Untreated wells were also serum starved in SFM. After 24 h, supernatants were collected and quantified by Luminex multiplex bead assay according to the manufacturer's instructions (Luminex assay by Celia Murciano). The trimeric gp140 glycosylated proteins (UG21 and ZM96) were produced by mammalian cell transfection or stably expressing lines rather than in bacteria or insect cells (like gp120 BAL and CN-54). UG21 was a kind gift from Dr Simon Jefferis (Imperial College London).

2.10 Global gene profiling by microarray analysis

5 x 10⁵ cells were exposed to HIV-1 YU2-R5 and LAI-X4 for an 8 h stimulation. The cells were washed with PBS and total RNA isolated using the GenElute total mammalian RNA miniprep kit (Sigma, UK). The following was performed by Chengguo Shen, a postdoctoral scientist and bioinformatician in the lab. Genomic DNA contamination was removed from the RNA sample using the Turbo DNase free kit (Ambion, UK). RNA was amplified using the MessageAmp Premier RNA Amplification Kit (Ambion, UK) then hybridized onto U133a 2.0 gene chips (Affymetrix, UK) after fragmentation. Chips were scanned (Affymetrix GeneChip Scanner 3000) and checked using Affymetrix Command Console (AGCC) software suite. This data was statistically analysed using Partek Genomics Suite (version 6.4, Partek, USA). The data was then assessed by myself. Gene lists representing up and down regulated genes were compared using the Venn diagram program Venny (Oliveros, 2007) and submitted to the Database for Annotation, Visualisation and Integrated Discovery (DAVID) (Huang da et al., 2009) and examined for gene ontology.

2.11 Fungal culture and quantification

The fungal wild-type strains used in this study were *C. albicans* SC5314 (Gillum et al., 1984), *C. albicans* 529L (in-house clinical isolate), *C. dubliniensis* (CD36), *C. tropicalis* (ATCC 750), *C. glabrata* (ATCC 2001), *C. guilliermondii* (in-house clinical isolate), *C. krusei* (clinical isolate (Jayatilake et al., 2008)) and *S. cerevisiae* (NCPF 3139). This represents a selection of strains of *Candida* species with one non-*Candida* fungal species. The *C. albicans* null mutant strains used in this study were yeast locked Δ eed1 (Zakikhany et al., 2007), pseudohyphal Δ tup1 (Braun and Johnson, 1997), and hyphal Δ nrg1 (Murad et al., 2001), as well as Δ pmr1 (Bates et al., 2005), Δ och1 (Bates et al., 2006) and Δ mnt1/2 (Munro et al., 2005), which are mutant in genes governing N- and O- glycosylation of *Candida* surface proteins. The parent strain of these mutants was *C. albicans* CAI4, which had the Ura3 gene replaced using the plasmid Clp10 (Murad et al., 2000), *C. albicans* CAI4 is a derivative strain of *C. albicans* SC5314. Particulate β -glucan was a kind gift from David Williams, University of Tennessee. Chitin was crab chitin (Sigma).

Fungal cultures were grown overnight at 30°C in 5 ml YPD (1% yeast extract, 2% peptone, 2% dextrose) with 5 μ l of a previous culture or from a frozen bead stock kept at -80°C. A 1 mL aliquot of the culture was centrifuged for 1 min at 14,500-15,000 rpm and resuspend in 1 mL filter sterilized PBS and washed twice. Using a spectrophotometer, a 1:100 dilution of the resuspended culture was measured at OD 600 nm to determine the

colony forming units (CFU)/mL using a standard curve previously established in the group. Then the fungal cells were diluted accordingly to obtain a concentration of 1×10^5 yeast/mL for the assay.

2.11.1 HIV-1 transfer from fungal cells to TZM-bl reporter cells

10^5 fungal cells from an overnight culture at 30°C were aliquoted into a 1.5 mL tube and resuspended with DMEM 10 with or without virus, as indicated (either YU2-R5 or LAI-X4 at MOI of 1, in 1 mL total volume). Samples were incubated overnight at 37°C on a gently shaking platform to allow for the virus to bind to the fungal cells. Meanwhile, TZM-bl cells (1×10^4 cells/well) were also seeded overnight (96-well plates). The fungal cells were washed three times with cold PBS and the pellets resuspended in 600 μ L (per sample tube) of DMEM 10 with addition of 34 μ L Fungizone/mL or 34 μ L 250 μ g/mL Amphotericin B (final concentration 0.136 μ g/ml) (PAA laboratories) to inhibit fungal growth and the killing of TZM-bl indicator cells by the fungus. The resuspended fungal pellets were then incubated with the TZM-bl cells. After 18-24 h incubation at 37°C, the cells were fed with 50 μ L of fresh medium containing Amphotericin B as described above. After a further 48 h, virus supernatant was removed and the cells were fixed with 0.05% glutaraldehyde for 10 min at room temperature and washed twice with PBS. Expression of β -galactosidase was determined by staining cells with X-Gal stain, as described in section 2.3.3, and incubating culture plates at 37°C for 1 h. Virus transfer was counted in HIV-1 yeast exposed wells and compared to control wells incubated with yeast not exposed to HIV-1.

Chapter 3 Expression of HIV-1 related receptors in oral and vaginal epithelial cells

3.1 Introduction

The initial step of HIV-1 infection of any host cell is surface receptor binding. Classical binding of HIV-1 to permissive host cells is the sequential interaction of the viral envelope protein gp120 with the cellular CD4 receptor and then a fusion co-receptor. For macrophages and T cells these fusion co-receptors are the chemokine receptors CCR5 (R5-trophic virus) or CXCR4 (X4-trophic virus), respectively (Pierson and Doms, 2003). Together, these receptors (CD4, CCR5/CXCR4) are termed canonical HIV-1 receptors. Despite these receptor dependencies, HIV-1 gp120 may also bind CD4⁺ cells such as spermatozoa (Baccetti et al., 1994), fibroblasts (Tateno et al., 1989) and oligodendrocytes (Harouse et al., 1991). Therefore, it is clear that HIV-1 utilizes several alternative receptor mechanisms for binding host cells that appear to be independent of the CD4/CCR5 and CD4/CXCR4 mechanism. These alternative, non-canonical host receptors include DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) (Geijtenbeek et al., 2000), ICAM-1 (intercellular adhesion molecule-1) (Paquette et al., 1998), GalCer (galactosylceramide) (Alfsen and Bomsel, 2002; Delezay et al., 1997; Harouse et al., 1991; Yahia et al., 1995), and heparan sulphate proteoglycans (HSPGs) such as syndecan-1 and -4 (Bobardt et al., 2003; Hamon et al., 2004; Urbinati et al., 2009; Wu et al., 2003) (see Chapter 1, literature review, sections 1.5, and Tables 1.2, 2.1 for details).

The vast majority of reports indicate that oral and vaginal epithelial cells do not express or express very little CD4 (Bobardt et al., 2007; Dezzutti et al., 2001; Kumar et al., 2006; Liu et al., 2003; Quinones-Mateu et al., 2003; Vacharaksa et al., 2008) and express CCR5 and CXCR4 at either undetectable or very low levels (Bobardt et al., 2007; Giacaman et al., 2007; Liu et al., 2003; Vacharaksa et al., 2008). However, since HIV-1 is known to bind epithelial cells from these mucosal sites (Dezzutti et al., 2001; Tugizov et al., 2011; Wu et al., 2003), it is thought HIV-1 gp120 also utilizes several alternative receptor mechanisms for binding epithelial cells at these mucosal sites. These include GalCer and HSPGs, which are commonly expressed on epithelial cells and are thought to promote HIV-1 binding (Bobardt et al., 2007; Wu et al., 2003).

In this chapter we aimed to determine the expression of canonical HIV-1 receptors (CD4, CCR5 and CXCR4) along with non-canonical HIV-1 receptors (DC-SIGN, HSPG's, ICAM-1 and GalCer) in three epithelial cell lines representing oral (TR146 (Rupniak et al., 1985) and FaDu (Rangan, 1972)) and vaginal (vulval - A431 (Giard et al., 1973) mucosal sites. This was performed to determine whether the presence of these HIV-1

receptors correlated with the ability of HIV-1 to bind (Chapter 4) and subsequently infect (Chapter 5) these epithelial sites, as this may provide clues as to why oral transmission of HIV-1 is a rare event in comparison with vaginal transmission. We utilized epithelial cell lines representative of two oral sites that have not previously been investigated with regard to oral HIV transmission but are one of the first cell types that come into contact with HIV-1 in the oral cavity: buccal (TR146) and oro-pharyngeal (FaDu) epithelial cells, both of stratified squamous cell origin. Comparison of these three cell lines also allowed for convenient and direct comparisons to be made with regard to receptor expression. We demonstrate that there are small but definitive differences in HIV-1 related receptor expression between oral and vaginal epithelial cells, which may result in differential ability of HIV-1 to bind these mucosal sites (Chapter 4 and 5).

3.2 Methods

3.2.1 mRNA expression

Methods are detailed in section 2.4.2. Briefly, RNA was isolated from the same number of plated resting cells. RNA was treated with DNase and converted to cDNA using HIV reverse transcriptase and then assayed with primers in Table 2-1 (for CD4, CXCR4, CCR5, DC-SIGN, SDC-1, SDC-4, ICAM-1 and β -actin) under cycling conditions in Table 2-2 in a Sybr green mastermix by realtime PCR.

3.2.2 Flow cytometry for surface protein expression

Methods are as detailed in section 2.4.1. Briefly cells were detached with mild EDTA-treatment, washed and stained with antibodies against CD4, CXCR4, CCR5, DC-SIGN, HSPGs, ICAM-1 and GalCer and washed and analysed by flow cytometry.

3.3 Results

The expression of epithelial cell receptors that bind HIV-1 gp120 can be determined at the mRNA and protein levels. In this study, we utilized quantitative reverse transcription PCR (qRT-PCR) to assess mRNA expression and flow cytometry to assess protein levels of CD4, CCR5, CXCR4, DC-SIGN, HSPG's (syndecan-1-4), ICAM-1 and GalCer. It should be noted that GalCer is a glycosphingolipid (non-protein moiety) and could not be investigated by mRNA expression.

3.3.1 Establishment of a qRT-PCR protocol to assess mRNA expression of HIV-1 related receptors

Prior to determining the mRNA expression levels of HIV-1 related receptors in epithelial cells, preliminary experiments were first undertaken to optimize the qRT-PCR protocol. A number of forward and reverse primer sets and cycling times were tested for each target gene (*CD4*, *CCR5*, *CXCR4*, *DC-SIGN*, *SDC-1*, *SDC-4* and *ICAM-1*) and various parameters were assessed including the dynamic range (5-log dilution series with a slope of approximately $-3.3 \pm 10\%$), R^2 value (a statistical term that indicates how good one value is at predicting another; an R^2 value >0.95 provides good confidence in correlating two values and a value >0.99 provides excellent confidence), precision (low standard deviation ≤ 0.250) and sensitivity (capable of effectively amplifying low copy numbers of starting template). Based on these assessment parameters, the optimal primer sets were chosen for detecting gene expression of the target genes. For all primer sequences and cycling conditions tested please refer to Chapter 2 (Materials and Methods, Section 2.4.2, Tables 2.1 and 2.2 respectively).

3.3.1.1 Gene expression of HIV-1 related receptors in oral and vaginal epithelium

Once the conditions for the qRT-PCR protocol were optimised, we proceeded with determining the expression of *CD4*, *CCR5*, *CXCR4*, *DC-SIGN*, *SDC-1*, *SDC-4* and *ICAM-1* in the oral (TR146, FaDu) and vaginal (A431) epithelial cell lines. This was performed in resting epithelial cells to determine the basal gene expression levels of these receptors, as under normal conditions (e.g. natural infection with HIV-1) epithelial cells are in the resting state when exposed to HIV-1. TZM-bl cells were included as a positive control, which are a HeLa-derived cell line (Derdeyn et al., 2000) that have been engineered to express CD4, CCR5 and CXCR4 (Platt et al., 1998).

To determine basal gene expression levels of these receptors, the total RNA was isolated from resting TR146, FaDu and A431 epithelial cells (see Chapter 2, Materials and Methods, section 2.4.2) and *CD4*, *CCR5*, *CXCR4*, *DC-SIGN*, *SDC-1*, *SDC-4* and *ICAM-1* gene expression determined using the optimised qRT-PCR protocol. The data are shown in Table 3.1 and are representative of between 5 and 10 independent experiments, each performed in technical triplicates with the average value for each technical triplicate presented.

The arbitrary copy number for each target gene is compared with *β -actin*, a housekeeping gene, used as a control, which was given an arbitrary mRNA copy number of 10000. Outlier quantified values more than two standard deviations from the mean were excluded from analysis (E). For direct comparisons between cell lines, the average arbitrary mRNA copy number for each gene is also presented in Fig 3.1.

Table 3-1: SyBrGreen qRT-PCR detection of HIV-1 related receptor genes in oral (TR146, FaDu), vaginal (A431) and TZM-bl cell lines.

Independent repeat ^a	Canonical HIV receptors				HIV binding factors			
	β -actin ^b	Co-receptors			DC-SIGN ^c	HSPG		ICAM-1 ^c
		CD4 ^c	CCR5 ^c	CXCR4 ^c		SDC-1 ^c	SDC-4 ^c	
TZM-bl-1	10000	2640	E	420	8	3100	E	4000
TZM-bl-2	10000	2040	1870	220	0	1300	E	3400
TZM-bl-3	10000	2090	7460	1120	0	900	28	600
TZM-bl-4	10000	1450	6670	430	0	820	31	700
TZM-bl-5	10000	E	3700	690	0	1320	34	1100
Average	10000	2055	4925	576	2	1488	31	1960
+/- SD	0	486	2253	347	4	929	3	1613
TR146-1	10000	E	E	E	0	2500	E	2600
TR146-2	10000	0	0	1	0	E	E	2900
TR146-3	10000	38	0	1	0	910	1	1659
TR146-4	10000	0	0	0	17	2011	0	5874
TR146-5	10000	0	0	0	0	1692	0	1306
TR146-6	10000	0	0	0	0	1093	0	2901
TR146-7	10000	2	0	0	0	1915	0	1001
TR146-8	10000	0	0	0	0	2164	0	2838
TR146-9	10000	21	0	0	0	842	0	2115
TR146-10	10000	74	0		2	1846	0	554
Average	10000	15	0	0	2	1664	0	2350
+/- SD	0	26	0	1	5	585	0	1578
FaDu-1	10000	0	0	10	E	E	100	1400
FaDu-2	10000	0	10	24	E	E	600	0
FaDu-3	10000	0	0	20	0	2020	100	1400
FaDu-4	10000	0	0	20	5	2080	100	1700
FaDu-5	10000	0	0	10	0	1040	200	1500
Average	10000	0	2	17	2	1713	220	1500
+/- SD	0	0	4	9	3	584	217	141
A431-1	10000	E	0	0	E	E	100	2500
A431-2	10000	E	0	1	E	2300	100	700
A431-3	10000	177	0	0	0	3728	125	127
A431-4	10000	16	0	0	0	8766	299	851
A431-5	10000	353	0	0	4	6361	249	302
A431-6	10000	66	E	0	11	2675	57	237
A431-7	10000	268	0	0	1	6103	213	639
Average	10000	179	0	0	3	4989	163	765
+/- SD	0	139	0	0	5	2513	90	810

^a Data are representative of between 5 – 10 independent experiments

^b Actin is a housekeeping control gene, which was given an arbitrary mRNA copy number of 10000.

^c Arbitrary mRNA copy number of target genes relative to β -actin.

SDC-1, syndecan-1; SDC-4, syndecan-4.

E: eliminated from analysis due to greater than 2 standard deviations from the mean.

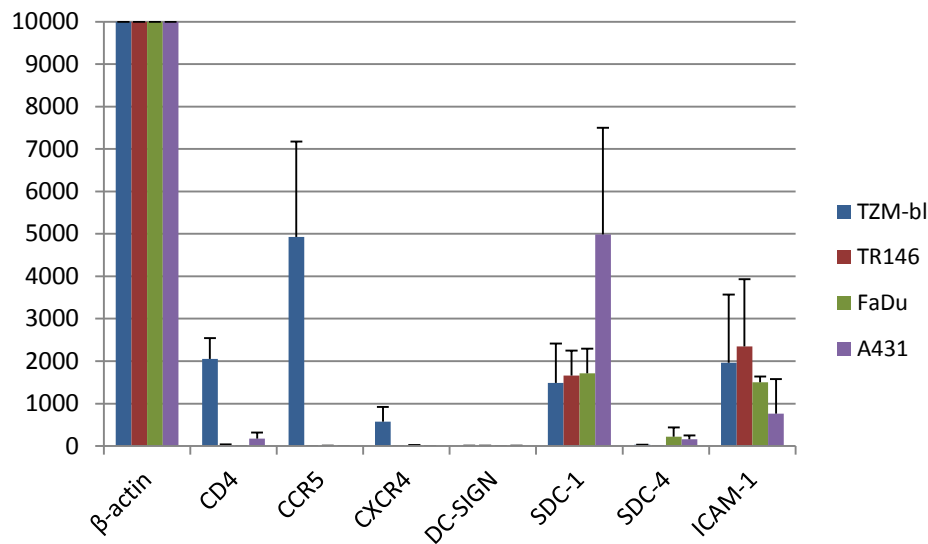


Figure 3-1: Averages mRNA transcript levels (number of copies) of HIV-related receptors in TR146, FaDu, A431 and TZM-bl cell lines, relative to 10000 copies β-actin. TR146 are shown in blue, FaDu in red, A431 in green, TZM-bl in purple.

The data presented in Table 3.1 and Figure 3.1 shows both similarities and differences in gene expression of HIV-1 related receptors between the two oral epithelial cell lines and between the oral and vaginal cell lines. With regard to the oral epithelial cell lines, both TR146 and FaDu cells expressed undetectable or very low levels of the canonical HIV-1 receptors *CD4*, *CCR5* and *CXCR4*. Although FaDu cells appear to express greater levels of *CXCR4* than TR146 cells, expression is very low compared with the control TZM-bl cells. Likewise, although TR146 cells appear to express greater levels of *CD4* than FaDu cells, *CD4* expression in TR146 cells is variable and, when detected, is present in very low amounts. Both TR146 and FaDu cells express similar (high) levels of the non-canonical receptors *SDC-1* and *ICAM-1*, with neither cell type expressing detectable amounts of *DC-SIGN*. The main difference between the two oral epithelial cell lines is that FaDu cells express greater levels of *SDC-4* compared with TR146 cells.

Like TR146 and FaDu oral epithelial cells, A431 vaginal epithelial cells expressed undetectable or extremely low amounts of *CCR5*, *CXCR4* and *DC-SIGN*. Interestingly though, unlike TR146 and FaDu oral cells, A431 vaginal cells expressed *CD4* mRNA, albeit at levels lower than the positive control TZM-bl cell line. A431 cells also expressed slightly elevated levels of *SDC-1* and reduced levels of *ICAM-1* compared with TR146 and FaDu cells, and similar levels of *SDC-4* as FaDu cells, which was absent in TR146 cells.

To summarize, gene expression studies indicate that resting oral epithelial cells do not express high levels of the canonical HIV receptors *CD4*, *CCR5* or *CXCR4*.

However, unlike buccal (TR146) oral epithelial cells, pharyngeal (FaDu) cells additionally express *SDC-4*. Notably, vaginal (A431) epithelial cells express *CD4* at low levels and *SDC-4* at similar levels as pharyngeal (FaDu) epithelial cells. Finally, *SDC-1* and *ICAM-1* are generally highly expressed in all epithelial cell types and *DC-SIGN* is undetectable.

3.3.2 Establishment of a flow cytometry protocol to assess surface expression of HIV-1 related receptors

The gene expression studies were useful in providing indicative information of which HIV-1 related receptors were expressed in resting epithelial cells. However, gene expression levels may not correlate with expression of these receptors on the epithelial cell surface, which is where HIV-1 would initially bind to epithelial cells during natural exposure to the virus. Therefore, it was important to verify whether gene expression levels correlated with surface protein expression of these receptors. To determine this we used flow cytometry to detect *CD4*, *CCR5*, *CXCR4*, *DC-SIGN*, *HSPGs* and *ICAM-1* on the surface of TR146, FaDu and A431 epithelial cells, and compared this with the positive control TZM-bl cell line. We also investigated the surface expression of GalCer as this glycosphingolipid moiety could not be investigated by gene expression. It should also be noted that the antibody used to determine surface expression of HSPG's detected all four syndecans (1-4), rather than just syndecan-1 and -4 as assessed in the gene expression studies. This was because we wanted to ensure that we could detect general HSPG expression on the epithelial cell surface as opposed to just specific HSPG's.

Prior to determining the expression of HIV-1 related receptors on the surface of epithelial cells, preliminary experiments were first undertaken to optimize the flow cytometry protocols. Section 2.4.1 shows which antibodies and respective dilutions were optimal to detect the expression of HIV-1 related receptors on the surface of epithelial cells.

3.3.2.1 Protein expression of HIV-1 related receptors in oral and vaginal epithelium

Once the flow cytometry protocol was optimized, we proceeded with determining the surface expression of the HIV-1 related receptors in resting TR146, FaDu and A431 epithelial cells. To achieve this, epithelial cells were isolated from monolayer cultures and labelled with antibodies specific to each target receptor (Chapter 2, section 2.4.1) and detected with a FITC-labeled secondary antibody (section 2.4.1) except for *ICAM-1* antibody that was directly conjugated to PE. Table 3.2 and Figure 3.2 represent the same data set and depict the surface protein expression of HIV-1 related receptors on TR146, FaDu and A431 epithelial cells in comparison to the positive control TZM-bl cells.

In most cases the data are representative of two independent experiments, each performed in technical duplicates with the average value for each technical duplicate presented. The data was obtained in addition to and as verification of data included in Kohli *et al* (2012, submitted manuscript).

Table 3-2: Surface protein expression of HIV related receptors on epithelial cells

	% of epithelial cells expressing HIV-1 related receptors						
	CD4 (+/-SD)	CCR5 (+/-SD)	CXCR4 (+/-SD)	DC-SIGN (+/-SD)	GalCer (+/-SD)	HSPGs ^a (+/-SD)	ICAM-1 (+/-SD)
TZM-bl	56 (9.4)	40 (7)	93 (3.5)	0.3 (0.1)	6.2 (3.2)	0(1.3)	96 (1.4)
TR146	1.2 (3.1)	13 ^b	5.8 ^b	0.1 (0.9)	28 (24)	4.9 (4.5)	99 (0.3)
FADU	0.7 (0.6)	2 ^b	2.5 ^b	0.2 (0.4)	18 (5.8)	2.5 (0.2)	62 (15)
A431	1.3 (3.7)	11 ^b	8 ^b	0.2 (0.4)	36 (13)	37 (16)	90 (6.7)

^a HSPGs; antibody detecting syndecans 1-4.

^b Represents singleton data points, thus no SD is shown. All other data sets are representative of two independent experiments

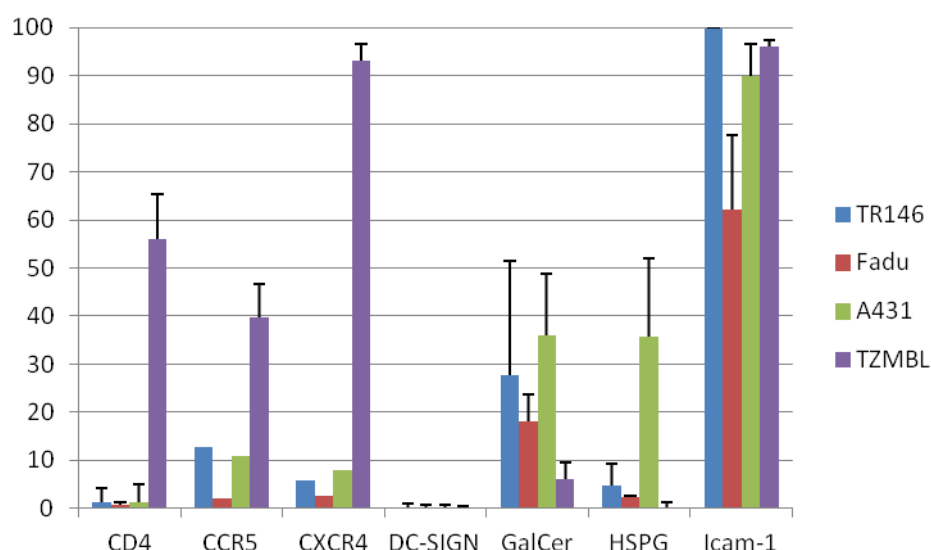


Figure 3-2: Percent surface protein expression of HIV-1 related receptors in TR146, FaDu, A431 and TZM-bl, cell lines as determined by flow cytometry using monoclonal primary antibodies for listed cell surface moieties. Primary antibodies were detected with goat anti-mouse

IgG conjugated with fluorescein isothiocyanate (FITC). The only directly conjugated ab was ICAM-1 to PE.

Both TR146 and FaDu oral cells expressed undetectable or low levels of CD4, CCR5 and CXCR4 on the surface, which supports the gene expression data. Although a greater number of TR146 cells appeared to express CCR5 than FaDu cells (13% versus 2%, respectively), expression was still low compared with the positive control TZM-bl cells (40%). A high percentage of TR146 and FaDu cells expressed ICAM-1 (99% versus 62%, respectively), but neither cell type expressed detectable amounts of DC-SIGN, again correlating well with the gene expression data. However, both TR146 and FaDu cells appeared to express minimal amounts of HSPGs, which was in contrast to the gene expression data that indicated the presence of SDC-1 mRNA for TR146 and both SDC-1 and -4 mRNA for FaDu cells. Given that the HSPG antibody detects syndecans 1-4, this was an unexpected result. This would suggest that although SDC-1 and SDC-4 are expressed at the mRNA level, they may not to be translated into surface protein expression. Finally, a similar percentage of TR146 and FaDu cells expressed the glycosphingolipid GalCer (28% versus 18%, respectively). Together, our data suggest that there were no major differences between buccal and oro-pharyngeal epithelial cells with regard to surface expression of the target HIV-1 receptors. Both cell types expressed undetectable/very low levels of the canonical HIV-1 receptors (CD4, CCR5 and CXCR4) but greater amounts of the non-canonical HIV-1 receptors (ICAM-1 and GalCer).

Like TR146 and FaDu oral epithelial cells, A431 vaginal epithelial cells expressed undetectable or very low amounts of CD4, CCR5, CXCR4 and DC-SIGN. This correlates with the gene expression data except for CD4, which was shown to be expressed by A431 cells, albeit at low levels. This would suggest that although CD4 is expressed at the mRNA level in vaginal epithelial cells, it appears not to be translated into surface protein expression (i.e. similar to HSPG's (SDC-1 and SDC-4) for oral epithelial cells). A similar percentage of A431 cells also expressed ICAM-1 (90%) and GalCer (36%) as the two oral epithelial cell lines. The only major difference between the vaginal and oral epithelial cells was that HSPG's were expressed on the surface of a good percentage of A431 cells (37%) but only minimally on TR146 (4.9%) and FaDu (2.5%) oral cells.

To summarize, surface protein expression studies indicate that resting oral and vaginal epithelial cells do not express significant levels of the canonical HIV receptors CD4, CCR5 or CXCR4, or the non-canonical receptor DC-SIGN. However, oral and vaginal epithelial cells do express surface ICAM-1 and GalCer, with vaginal cells additionally expressing surface HSPG's.

3.4 Discussion

The majority of HIV-1 infections worldwide are acquired via mucosal surfaces, predominantly across the female or male genital tracts (Hladik and McElrath, 2008). In contrast, HIV-1 transmission through the oral mucosa is thought to be uncommon (Cohen, 2008; Jotwani et al., 2004). However, studies in primates indicate that oral transmission is a possible occurrence since non-traumatic oral exposure to SIV results in regional dissemination followed by systemic infection (Baba et al., 1996; Joag et al., 1997; Milush et al., 2004). Given that both genital and oral mucosae are lined by epithelial cells, this raises the possibility that in addition to representing a barrier to HIV-1 transmission at one level, epithelial cells may also be a conduit for viral entry. This is particularly important given the occurrence of viral transmission during heterosexual intercourse, during oro-genital contact in adults, and in nursing infants. In this regard, binding of HIV-1 to epithelial cells could be an essential first step in subsequent viral transmission across the mucosae.

HIV-1 has been shown to utilize many surface receptors to bind to host cells including the classical receptors CD4, CCR5 and CXCR4 as well as the alternative receptors DC-SIGN, HSPG's, ICAM-1 and GalCer (Cook et al., 1994; Fantini et al., 1993; Fortin et al., 1998; Geijtenbeek et al., 2000; Paquette et al., 1998; Saidi et al., 2007; Tan et al., 1993; Yahia et al., 1995). Therefore, in this chapter we aimed to determine the expression of CD4, CCR5, CXCR4, DC-SIGN, HSPG's, ICAM-1 and GalCer in three epithelial cell lines representing oral buccal (TR146), oro-pharyngeal (FaDu) and vaginal (vulval - A431) mucosal sites. This was performed to determine whether these HIV-1 related receptors were differentially expressed at these body sites, which may provide clues as to why oral transmission of HIV-1 is considered a rare event in comparison with vaginal transmission. We demonstrate that there are small but definitive differences in HIV-1 related receptor expression between oral and vaginal epithelial cell lines that may, if true for primary epithelia, ultimately result in the differential ability of HIV-1 to bind and/or subsequently infect these mucosal sites, which was investigated in Chapters 4 and 5.

3.4.1 Expression of canonical HIV-1 receptors in oral epithelial cells

Both the gene and protein expression studies indicated that resting TR146 and FaDu oral epithelial cells express generally undetectable levels of CD4 and very low levels of CCR5 and CXCR4 (Tables 3.1, 3.2 and Figures 3.1, 3.2). Although these cell lines have not previously been used in HIV-1 studies, a number of studies using epithelial cells isolated from various sites within the oral cavity have been undertaken.

Our data are in line with the majority of these studies, which also indicate a general lack or low level expression of CD4, CCR5 and CXCR4 (Chapter 1, Literature review, Section 1.6.1, Tables 1.1 and 1.2) (Giacaman et al., 2008; Kumar et al., 2006; Liu et al., 2003; Quinones-Mateu et al., 2003; Vacharaksa et al., 2008). However, several other studies have demonstrated the presence of CD4, CCR5 and CXCR4 in oral epithelial cells (Moore et al., 2002; Moore et al., 2003; Tugizov et al., 2011).

Moore *et al.* demonstrated that two salivary gland epithelial cell lines expressed *CXCR4* mRNA, one expressed *CCR5* mRNA, but both were negative for *CD4* mRNA, and that this correlated with surface protein expression (Moore et al., 2002). The same authors also demonstrated *CCR5* and *CXCR4* mRNA expression in two keratinocyte and two adenoid epithelial cell lines, with one adenoid cell line expressing *CD4* mRNA, which again correlated with protein expression except surface CD4 was not detected (Moore et al., 2003). In addition, Giacaman *et al* (2007) showed that immortalized TERT-2 cells (human oral keratinocyte line) expressed *CXCR4* and to a lesser extent *CCR5* mRNA and surface protein, whereas another study demonstrated weak expression of *CD4*, *CCR5* and *CXCR4* mRNA in seven gingival keratinocytes biopsies and five palatine tonsil epithelial samples (all primary cells) by microarray analysis (Moutsopoulos et al., 2007). However, the unit for the expression level was not provided in the latter paper, making the expression data difficult to evaluate. Other studies only investigated surface protein expression of CD4, CCR5 and CXCR4. These studies also demonstrated the general absence of surface CD4 concomitant with the absence or low level expression of CCR5 and CXCR4 in primary oral keratinocytes, immortalized TERT2 cells and adult/foetal tongue and tonsillar epithelium (Liu et al., 2003; Tugizov et al., 2011; Vacharaksa et al., 2008).

In summary, our data are in line with the published literature and we conclude that CD4, CCR5 and CXCR4 are absent or expressed at very low levels in oral epithelial cell lines. Given that CD4, CCR5 and CXCR4 are required for efficient viral infection of immune cells (macrophages and T-cells (Alkhatib et al., 1996; Berson et al., 1996; Klatzmann et al., 1986), this suggests that oral epithelial cells are unlikely to support productive HIV-1 infection (see Chapter 5).

3.4.2 Expression of non-canonical HIV-1 receptors in oral epithelial cells

Gene expression studies indicated that *SDC-4* was preferentially expressed in pharyngeal (FaDu) but not buccal (TR146) epithelial cells, and that *SDC-1* and *ICAM-1* were highly expressed in both oral epithelial cell types (Table 3.1, Figure 3.1). Protein studies demonstrated surface expression of ICAM-1 and GalCer but that HSPG

expression (SDC1-4) was surprisingly very low. Notably, FaDu cells expressed higher levels of ICAM-1 and GalCer compared with TR146 cells. DC-SIGN was generally undetectable by mRNA or protein (Table 3.1, 3.2, Figures 3.1, 3.2).

Our data are generally in line with the current literature demonstrating the presence of GalCer and HSPGs in oral epithelial cells (Chapter 1, Literature review, Section 1.6.1.2, Table 1.2). The most common non-canonical HIV-1 receptor analysed is GalCer, which has been detected in nearly all epithelial cell types when investigated, including salivary gland epithelial lines, primary gingival keratinocytes and adult/foetal tongue and tonsil epithelium (Liu et al., 2003; Moore et al., 2002; Moore et al., 2003; Tugizov et al., 2011). However, one study in immortalized TERT-2 cells showed little (<1%) GalCer on the surface (Vacharaksa et al., 2008), which opposes the above studies and our findings. The Vacharaksa *et al* (2008) study also demonstrated high levels of surface HSPGs (91% \pm 20%), which again contrasted with our data (4.9% \pm 4.5% for TR146, and 2.5% \pm 0.2% for FaDu) and that of Tugizov *et al* (2011) (35-40 %) who investigated adult tongue epithelium, both of which demonstrated lower expression levels of surface HSPGs. This suggests that the TERT-2 cells may not be representative of epithelial cells within the oral cavity with regard to surface GalCer and HSPG expression, which may affect their relevance for HIV-1 infection studies of oral epithelial cells. Only one study has investigated DC-SIGN expression in oral epithelial cells, in a palatine tonsil explant, and was undetectable (Vacharaksa et al., 2008). We are unaware of any HIV-1 related studies investigating mRNA or protein levels of ICAM-1 in oral epithelial cells.

In summary, we conclude that oral epithelial cells express GalCer and ICAM-1, but limited amounts of HSPGs and an absence of DC-SIGN. Also, in the case of HSPGs (SDC-1 and SDC-4) mRNA expression may not translate into surface protein expression. It appears that epithelial cells from different sites within the oral cavity express variable levels of non-canonical HIV-1 related receptors and indicates that if HIV-1 binds to epithelial cells (see Chapter 4), it is most likely via non-canonical HIV-1 related receptors rather than CD4, CCR5 and CXCR4.

3.4.3 Expression of canonical and non-canonical HIV-1 receptors in vaginal epithelial cells

Like oral epithelial cells, vaginal (A431) epithelial cells expressed minimal levels of CD4, CCR5, CXCR4 and DC-SIGN, but higher levels of ICAM-1 and GalCer. As in the case of HSPGs in oral epithelial cells, *CD4* mRNA expression in vaginal epithelial cells appears not to be translated into surface protein expression. The main difference

between the two cell types was that vaginal cells expressed higher levels of surface HSPG's, unlike oral epithelial cells.

Although no studies could be found relating to gene expression of the HIV-1 related receptors in vaginal epithelial cells, several studies have investigated surface expression of these receptors in female genital tract epithelium (Chapter 1, Literature review, Section 1.6.1.2, Table 1.2). Asin *et al* (2003) examined two uterine (ECC1, RL95-2) and an endometrial (HEC-1A) epithelial line, which expressed CD4, CXCR4 and low levels of GalCer (Asin *et al.*, 2003). Saïdi *et al* (2007) also studied the HEC-1A line and found it to express high levels of CXCR4 and GalCer, with the GalCer finding contrary to that of the previous study (Saïdi *et al.*, 2007). Tugizov *et al* (2011) also detected high levels of GalCer on HEC-1A cells, but only moderate expression of HSPGs and low expression of CXCR4 and CCR5 expression, with CD4 being undetectable. Very few studies have investigated HIV-1 receptor expression in primary vaginal epithelial cells. Bobardt *et al* (2007) studied primary vaginal epithelial cells from four donors and demonstrated very low surface expression of CD4, CXCR4, CCR5 and GalCer but high levels of HSPGs, including SDC-1 and SDC-2. However, since only average mean fluorescence values were shown with no standard deviations, it is difficult to evaluate inter-sample variation in expression in this study. Most recently, in a cervical biopsy sample Tugizov *et al* (2011) detected moderate levels of surface CXCR4, CCR5 and HSPGs but low levels of GalCer, with no detectable CD4. These published studies suggest that vaginal epithelial cells generally express GalCer and HSPGs at high/moderate levels, low amounts of CXCR4 and CCR5 and low/undetectable levels of CD4, and that vaginal cell lines generally express higher levels of these receptors than primary vaginal epithelial cells. As with oral epithelial cells, we are unaware of any HIV-1 related studies investigating mRNA or protein levels of ICAM-1 in vaginal epithelial cells. Notably, A431 express less than ICAM-1 than the two oral epithelial cell lines, potentially indicating less tight intercellular adherence in this monolayer culture. However, the downstream biological effect of this observation with regard to HIV-1 infection is unknown.

In summary, our data are in line with the published literature and demonstrate that vaginal epithelial cell lines express minimal levels of CD4, CXCR4, CCR5 and DC-SIGN but increased levels of GalCer, HSPG's and ICAM-1. Furthermore, the increased amount of surface GalCer and HSPG's in vaginal epithelial cell lines as compared with oral epithelial cells may indicate that vaginal epithelial cells potentially have a greater capacity to bind HIV-1 via non-canonical HIV-1 related receptors than oral epithelial cells. This hypothesis was tested in Chapter 4.

3.4.4 General discussion

In this chapter we used real-time qRT-PCR and flow cytometry to determine mRNA and protein expression of HIV-1 related receptors in oral and vaginal epithelial cell lines. Most gene expression studies predominantly use semi-quantitative methods based on intensities of RT-PCR products. In this study we used real-time RT-PCR, which provides a more robust and quantitative measure of gene expression than most current studies in the literature. An additional strength of our approach is that all total RNA samples were first reverse transcribed together in equal concentrations prior to performing qPCR, which limits the variability that could be introduced by changes in efficiency between these two PCR steps. Therefore, in comparison with the published studies we are confident that data accurately reflect HIV-1 related receptor gene expression in epithelial cells. The flow cytometry protocols are in line with current approaches investigating surface protein expression of HIV-1 related receptors and our data adds support and substance to the current literature in this area.

It should be noted that we have used cell lines as models of normal human oral and vaginal tissues. The assumption made is that the tumour lines used approximate normal human tissues. However, many would argue that tumour cell lines are not representative of normal mucosal sites (Yadev et al., 2011; Moharamzadeh et al., 2012) and that any patterns observed in cell lines must be verified in primary cells. Tumour cell lines are much easier to obtain and culture and we did not have ethical approval for the collection of primary female genital tract epithelium. Confirmation of the findings in primary tissues would have been highly desirable but was deemed beyond the scope of this thesis.

We note that *SDC-2* (syndecan-2) and *SDC-3* (syndecan-3) gene expression was not assayed by qRT-PCR alongside *SDC-1* and *SDC-4*. This was because in the same study that suggested *SDC-4* forms complexes with CXCR4 on primary lymphocytes and macrophages, oligomerized and glycanated *SDC-2* (in contrast to *SDC-4*) could not be co-immunoprecipitated with CXCR4 (Hamon et al., 2004). Also, while *SDC-3* has been suggested to mediate HIV-1 attachment in dendritic cells (de Witte et al., 2007), *SDC-3* is very weakly expressed in human primary (genital) epithelial cells (Bobardt et al., 2007) and a role in HIV-1 attachment to epithelium has not been demonstrated. Given that our flow cytometry studies utilized an antibody that detected all four syndecans (*SDC-1-4*), we deemed it unnecessary to assess *SDC-2* and *SDC-3* mRNA levels by qRT-PCR.

While gene and surface protein expression data correlated well, this was not always the case (e.g. *SDC-1* and *SDC-4* mRNA expression in TR146 and FaDu cells but low HSPG surface protein expression, and presence of *CD4* mRNA transcripts in A431 vaginal cells but undetectable surface protein). However, it is well known that expression

of mRNA transcripts do not always reflect protein levels or that this provides any indication that the protein will be functional (Bustin et al., 2009). Given that our studies utilized cell lines, mRNA and surface protein expression levels were sometimes assessed at different passage number or different times of isolation. Eliminating this disparity may have perhaps minimized any differences in mRNA and protein detection.

We also stress that any differences between our data and those described in the literature may be partly explained by the type of epithelial cell used. For example, the FaDu epithelial cell line used in this study is derived from squamous epithelium of pharyngeal origin, whereas the adenoid studies (pharyngeal tonsil) in the literature are based on pseudostratified columnar epithelium. Since differences in epithelial origin may contribute to the variations in gene and surface protein expression of HIV-1 related receptors, this may complicate direct data comparisons between studies. Finally, it is recognised that our data are based on cell lines and that expression of HIV-1 related receptors may be different in primary buccal and pharyngeal squamous epithelial cells. This is particularly relevant given the heterogenous nature of most human populations and was recently highlighted in a study that showed variability in HIV-1 receptor expression across several tonsillar tissue sections isolated from different individuals (Kumar et al., 2006). Therefore, variability in expression patterns of HIV-1 related receptors between individuals may alter the ability of HIV-1 to bind epithelial cells during natural exposure to the virus *in vivo*. This may result in some individuals becoming more susceptible to HIV-1 attachment and subsequent infection than others.

3.5 General conclusions

In this chapter we set out to determine the levels of mRNA and surface protein expression for the HIV-1 receptor CD4, its co-receptors CXCR4 and CCR5, as well as several other alternate HIV-1 receptors and binding factors (DC-SIGN, HSPG's, GalCer and ICAM-1) in epithelial lines that we use as models of mucosal epithelia. We have quantified the expression for these HIV-1 related receptors in three epithelial cell lines and show that oral and vaginal epithelial cell lines express minimal levels of CD4, CCR5, CXCR4 and DC-SIGN, but higher levels of GalCer and ICAM-1. Vaginal epithelial cell lines additionally express increased levels of HSPG's on their surface. These small differences in HIV-1 related receptor expression between oral and vaginal epithelial cells may result in the differential ability of HIV-1 to bind and/or subsequently infect these mucosal sites. This was investigated in Chapter 4 (HIV-1 binding) and Chapter 5 (HIV-1 infection). Additional verification in primary normal cells would be desirable but was beyond the scope of the thesis.

Chapter 4 Binding of HIV-1 to oral and vaginal epithelial cells

4.1 Introduction

In the previous chapter, the mRNA gene expression and flow cytometry for surface receptor expression data suggested that there is potential for HIV-1 to bind to oral and vaginal epithelium according to our models. Although TR146 (buccal), FaDu (pharyngeal) and A431 (vaginal) cells express low surface levels of the HIV-1 receptor CD4 and co-receptors CCR5 and CXCR4, they do express the alternative HIV-1 receptor GalCer and HIV-1 binding factors HSPGs and ICAM-1. Therefore, all three epithelial cell lines possess molecules on their surface that have been documented to bind HIV-1. Although the evidence for direct interactions between HIV-1 and epithelial cells is often conflictory, there is a solid body of literature to suggest that HIV-1 directly binds epithelial cells.

Kage *et al* (1998) demonstrated that latex microparticles coated with gp120 were internalised by gingival epithelial cells, suggesting direct interaction with the virus particle envelope (Kage *et al.*, 1998). More recently, Rodriguez-Inigo (2005) used *in situ* hybridization of oral biopsies from HIV-1 positive patients to co-localize viral RNA with oral keratinocytes, which is also suggestive of direct interaction (Rodriguez-Inigo *et al.*, 2005). Two studies used GFP-vpr labeled HIV-1 to visualize HIV-1 in contact with epithelium. In 2009, Nittayananta *et al.* exposed differentiated oral epithelial organotypic cultures to GFP-labeled HIV-1 and observed the virus penetrating up to 15 µm into the model, although most GFP-positive particles remained on the apical surface (Nittayananta *et al.*, 2009). Tugizov *et al.* (2011) demonstrated similar results with adult tonsil biopsies, but due to low magnification it is difficult to observe direct interaction between the epithelial cell membranes and virus particles. Two additional electron microscopy studies also showed that HIV-1 infected peripheral blood mononuclear cell (PBMC) interact with intestinal (Alfsen *et al.*, 2005) and foreskin (Ganor *et al.*, 2010) epithelial cells but, again, in both studies it was difficult to visualize distinct virus particles attached to the epithelial surface. Thus, at the physical level there is strong, suggestive evidence of direct interactions between HIV-1 and epithelial cells but it has been difficult to visualize individual virus particles bound on the cellular level.

Other studies have explored inhibition of HIV-1 infection using antibodies and interfering molecules to understand which molecules are required for binding to epithelium. Antibodies against CD4 blocked infection of some colonic epithelial lines but not HT29 (Fantini *et al.*, 1991; Omary *et al.*, 1991). In addition, peptide portions of GalCer interfered with binding of gp120 to GalCer decreased HIV infection by 50% (Yahi

et al., 1995). Next, heparin was shown to be able to compete 90% of RF (X4) HIV binding, while heparanase also reduced association with uterine epithelium (Wu et al., 2003). Binding of HIV to primary vaginal epithelial cells was shown to be independent of CD4, CXCR4 and CCR5 using inhibitors and neutralising antibodies (Bobardt et al., 2007). Endometrial cells stripped of HSPG mostly abolished their interaction with X4 and R5 HIV versus controls (Saidi et al., 2007). In addition mannose receptors were implicated in HIV transcytosis since fucose, N-acetylglucosamine and mannosylated-residues inhibited the, interaction of HIV with epithelium (Saidi et al., 2007). Thus, alternative HIV binding molecules have been shown by interference studies to be responsible for HIV binding to epithelium.

Therefore, in this chapter we aimed to demonstrate whether HIV-1 R5 and X4 directly bind to oral and vaginal epithelial cell lines *in vitro*. This was investigated using two separate methods: detection of whole virus binding to epithelial cells by analysis of HIV-1 envelope gp120 protein by flow cytometry and presence of the HIV-1 capsid p24 by Western blot. We detected the presence of gp120 envelope on the surface of individual epithelial cells using two primary monoclonal antibodies and that this interaction was sensitive to trypsin. However, trypsin digestion did not completely abolish virus binding (our ability to detect gp120), suggesting that HIV-1 binds to oral and vaginal epithelial cell lines via both protein and non-protein surface moieties. In addition, protein extracts from HIV-1 exposed oral and vaginal epithelial cells also tested positive for HIV-1 p24 by Western blotting. In conclusion, our data suggest that oral and vaginal epithelial cell lines have the ability to directly bind and capture HIV-1.

4.2 Methods

4.2.1 Detection of HIV-1 gp120 by flow cytometry

The methods used are detailed in Chapter 2.4.3, briefly TR146, FaDu, A431 and TZM-bl cells were incubated with cell free YU2 (R5) or LAI (X4) infectious virus overnight (16 h) at 4°C and thoroughly washed with PBS. Surface gp120 was detected using F425 B4e8 and F425 A1g8 (Pantophlet et al., 2007, Bell et al., 2008), followed by detection with a Cy5-labeled secondary antibody.

4.2.2 Interference of detection of HIV-1 gp120 with trypsin digestion

The methods are described in detail in 2.4.4. Briefly the cell lines were exposed to HIV as described in the previous section but after washing, trypsin treatment for 5 min at 37°C was used to see if it would reduce our ability to detect cell surface bound gp120 (with F425 B4e8) (Pantophlet et al., 2007, Bell et al., 2008), by flow cytometry.

4.2.3 Western blot for p24 capsid on epithelial cells extracts

Epithelial cells were exposed to HIV-1 R5 (YU2) and X4 (LAI) viruses for 24 h at 37°C (Chapter 2, section 2.5.1 detection of p24), washed extensively with PBS and lysed for protein extraction. Samples were analysed by Western blot by probing for HIV capsid p24 using HIV-1 p24 Gag Monoclonal (#24-2) (from ARRRP) (Simon et al., 1997).

4.3 Results

4.3.1 Whole virus binding to epithelial cells: detection of HIV-1 gp120 by flow cytometry

In preliminary experiments, to investigate whether epithelial cells were able to bind and capture HIV-1 we tested a number of assay systems. Initially, a series of p24 antibodies (data not shown) and gp120 antibodies were used to determine whether a reliable cellular ELISA assay could be developed. However, the ELISA system we tried to develop (Abcam goat antibody to gp120 biotin conjugated, 53937) demonstrated high background staining of oral and vaginal epithelial cells making it impossible to distinguish virally exposed cells from untreated cells with confidence (data not shown).

Therefore, we next developed a flow cytometry assay for the detection of HIV-1 gp120 on the surface of epithelial cells. Two human monoclonal antibodies obtained from the NIH AIDS reagent program proved useful for the establishment of this assay: F425 B4e8 (ARRRP no. 7626) and F425 A1g8 (ARRRP no. 7624) provided by Dr. Marshall Posner and Dr. Lisa Cavacini (Pantophlet et al., 2007; Bell et al., 2008). The main reason for their selection was because they had broadly neutralizing characteristics (therefore would recognize many gp120s) and that a dilution was specified for flow cytometry applications on the data sheet, although other antibodies were initially assayed alongside these (see section 2.4.3 and Appendix C).

TR146, FaDu, A431 and TZM-bl cells were incubated with cell free YU2 (R5) or LAI (X4) virus overnight (16 h) at 4°C. The reduced incubation temperature enabled the direct visualization of surface bound HIV-1 in all cell types. Surface gp120 was detected using F425 B4e8 (see Figure 4.1 for representative experiment, Figure 4.2 for average of 2-3 experiments) and F425 A1g8 (Figure 4.3), followed by detection with a Cy5-labeled secondary antibody. Outlying data points (greater than 3 standard deviations from the mean) were eliminated from the analysis.

In Fig 4.1, the first column represents the unstained (no antibody) cell control for each of the cell lines. Gates were added (polygon shape) using the WinMDI program to encompass the unstained cells and this was taken to be 0% cells shifted towards Cy5 positive. The second column represents cells stained with primary and secondary

antibody (without HIV-1 addition), a second cell control for non-specific staining. The third and fourth columns represent gp120 detection of epithelial cells exposed to HIV-1 YU2 (R5) and HIV-1 LAI (X4), respectively. The percentage displayed on these plots represents the percent Cy5-fluorescence shift to the right versus the unstained cell control. However, to obtain a net number for percent of cells with bound HIV-1 on the surface, percentages for stained, untreated cells were subtracted from the percentages of virally exposed cells. Data are from 2-3 experiments are represented in Fig 4.2.

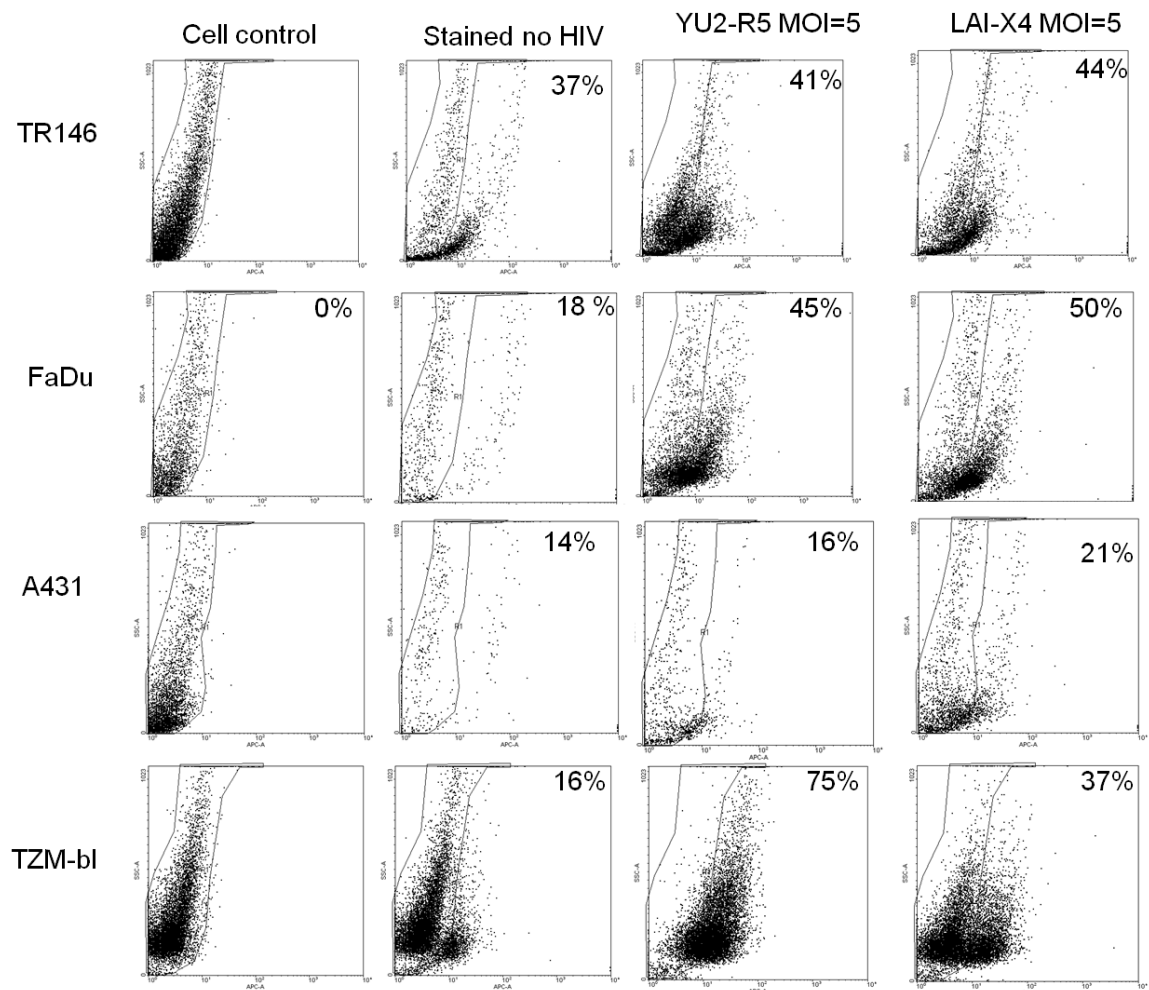


Figure 4-1: Detection of immobilized gp120 on the cell surface of oral and vaginal epithelial cells by flow cytometry. Oral (TR146, FaDu), vaginal (A431) and control (TZM-bl) cells were incubated overnight (16 h) at 4°C with infectious R5 and X4 HIV-1, washed, and epithelial surface-bound gp120 detected using human monoclonal antibody F425 B4e8 and a Cy5-labeled secondary antibody. The plots show a Cy5 fluorescence shift to the right (read on APC channel) after viral incubation. Percentages represent the proportion of cells shifted to higher fluorescence when compared with the cell control. Data are representative of three independent experiments.

This assay shows that both R5 and X4 gp120 can be detected on the surface of TR146, FaDu and A431 cells, demonstrating direct binding of infectious virus to oral and vaginal epithelial cells. With regard to the oral epithelial cell lines, FaDu cells appeared to capture both R5 and X4 virus more efficiently than TR146 cells: 27-32% versus 4-7%, respectively. Notably, A431 vaginal cells exhibited similar (low level) HIV-1 binding ability

as TR146 cells (2-7%). It should be noted that X4 virus can be detected to a slightly higher degree than R5 virus on the surface of all three epithelial cell lines despite the MOI prior to incubation being estimated to be equivalent. However, these differences are marginal. Interestingly, in the case of the positive control susceptible TZM-bl cells, R5 virus appears to bind better than X4 virus.

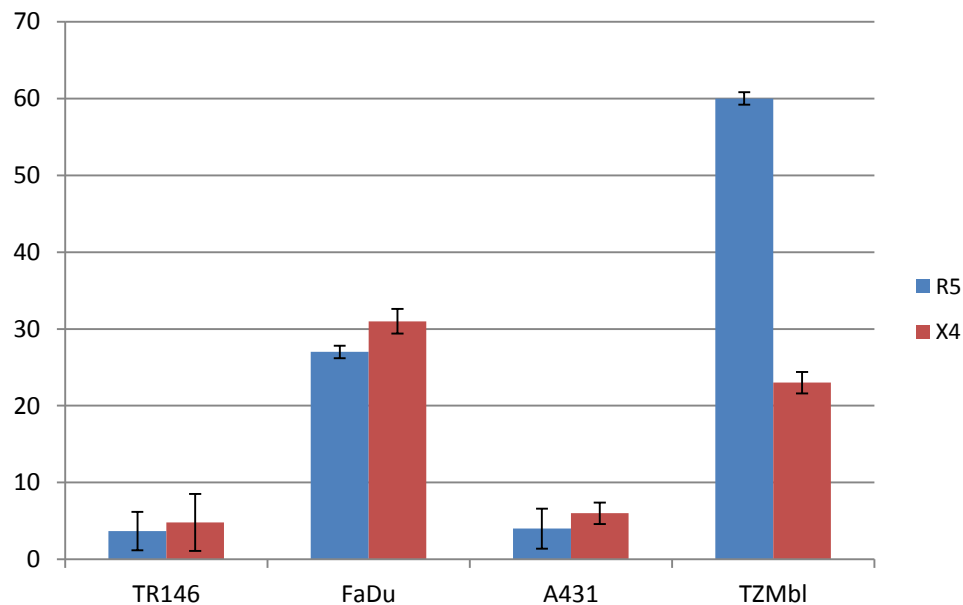


Figure 4-2: Detection of net immobilized gp120 on the cell surface by flow cytometry using a Cy5-labeled secondary to detect the human monoclonal antibody against HIV-1 gp120 F425 B4e8. Percentages are calculated by subtracting the Cy5 percentage for HIV untreated and antibody stained cells from the percent of cells shifted towards higher Cy5 fluorescence for HIV treated cells. The graph represents an average of 2-3 experiments.

To determine the reproducibility of the findings, data were repeated using a second gp120 primary monoclonal antibody (F425 A1g8; ARRRP no.7624). The data are presented in Figure 4.3 and reveals a globally similar percent HIV-1 binding profile as F425 B4e8 antibody (Fig 4.2). However, differences were observed between the two antibodies, which might be because F425 A1g8 recognises a different epitope on gp120 than F425 B4e8. First, HIV-1 YU2 R5 exhibits slighter higher binding to FaDu cells than HIV-1 LAI X4 (although this is a singleton data point) and, second, both R5 and X4 virus exhibit high binding to TZM-bl cells.

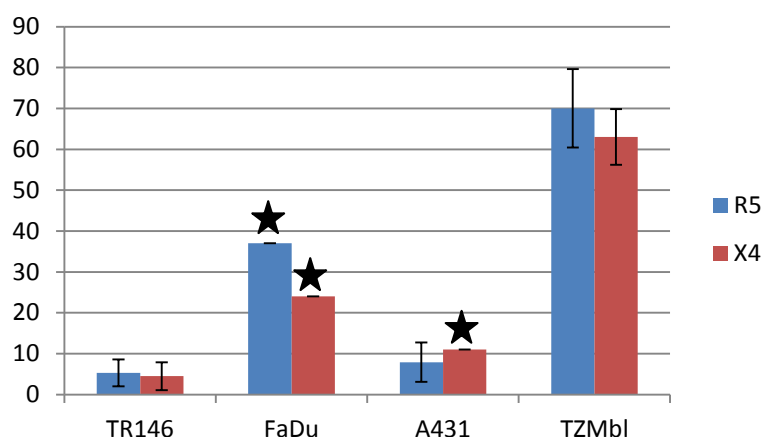


Figure 4-3: Detection of net immobilized gp120 on the cell surface by flow cytometry using a Cy5-labeled secondary to detect the human monoclonal antibody against HIV-1 gp120 F425 A1g8. Percentages are calculated by subtracting the Cy5 percentage for stained, untreated cells from the percent of cells shifted towards Cy5 for HIV treated cells minus the. The graph represents an average of 1-3 experiments. ★ Represents singleton data point.

In conclusion, the flow cytometry data indicates that there is generally low level HIV-1 binding to oral and vaginal epithelial cells. However, both R5 and X4 virus appear to bind pharyngeal epithelial cells (FaDu) more efficiently than buccal epithelial cells (TR146).

4.3.2 HIV-1 binding to oral and vaginal epithelial cells is partially trypsin sensitive

Since HIV-1 was capable of binding to epithelial cells, we wished to determine the nature of the interaction further. Trypsin treatment could provide information on whether the interaction between gp120 and epithelial cells was mediated by a trypsin-sensitive protein moiety or a non-protein surface moiety such as GalCer or the N-linked glycans (accounting for half the molecular mass of gp120 according (Allan et al., 1985).

TZM-bl, TR146 and FaDu cells were infected with either YU2 (R5) or LAI (X4) virus at an MOI of 5 overnight at 4°C. Cells were then treated with trypsin-EDTA for 5 min at 37°C before being blocked in PBS/10% BSA at room temperature. Any remaining HIV-1 bound to epithelial cells was detected using the HIV-1 gp120 monoclonal antibody F425 B4e8 by flow cytometry as described above (see Chapter 2, section 2.1.7.1 for full details). These experiments were performed on three separate occasions using two batches of F425 B4e8 antibody. Both batches provided similar data and a representative experiment is shown in Figure 4.4.

Trypsin treatment of virally exposed cells greatly reduced the ability to detect gp120 on all epithelial cell types as well as TZM-bl positive control cells. With TR146 cells, HIV-1 R5 binding was reduced by 78% after trypsin treatment, whereas HIV-1 X4 binding was more resistant and was only reduced by 32%. Notably, R5 binding was almost abolished in FaDu cells (greater than 95%) whereas X4 binding was reduced by 70%. Vaginal A431 cells demonstrated similar data to the oral TR146 cell line, with 75% reduction in R5 binding and only 15% reduction in X4 binding. Finally, the susceptible control TZM-bl reporter cells demonstrated 71% reduction in both R5 binding and X4 binding (62% to 12%) but notably over 10% of the originally detected virus remains detectable on surface of TZM-bl cells.

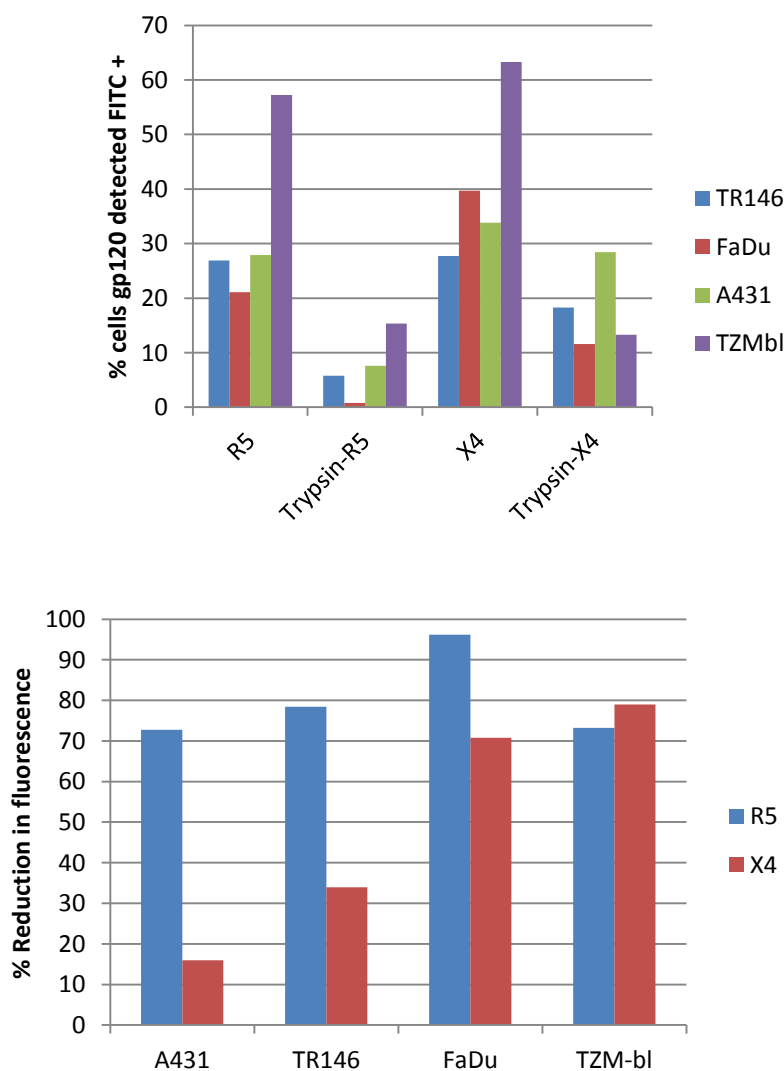


Figure 4-4: Reduction of detection of gp120 on the surface of virally exposed epithelial lines, treated with trypsin (versus untreated), by flow cytometry using a Cy5-labeled secondary to detect the human monoclonal antibody against HIV-1 gp120 F425 B4e8. Top panel represents percent positive versus secondary alone stained cells and trypsin treated exposed cells versus secondary alone, trypsin treated cells. The bottom panel percentage reductions are calculated by subtracting the Cy5 secondary alone stained virally exposed cells percentage from the primary and secondary stained and virally exposed cells. The graph shows

one representative experiment of 3 independent experiments with a new batch of anti-gp120 antibody (compared to Figures 4-1 to 4-3). R5: YU2-R5 virus. X4: LAI-X4 virus.

4.3.3 Detection of HIV-1 p24 on virally exposed epithelial samples

The flow cytometry data demonstrated that HIV-1 R5 and X4 virus was able to bind oral and vaginal epithelial cells. We wished to verify this data so a second assay was developed to detect the presence of the HIV-1 capsid protein p24 on the surface of epithelium after virus binding by Western blot.

In this experiment epithelial cells were exposed to HIV-1 R5 (YU2) and X4 (LAI) viruses for 24 h at 37°C (Chapter 2, section 2.1.8.1 for Western Blot for binding protocol). The exposed cells were washed extensively with PBS and lysed for protein extraction. Samples were analysed by Western blot by probing for HIV capsid p24 Gag monoclonal (Simon et al., 1997).

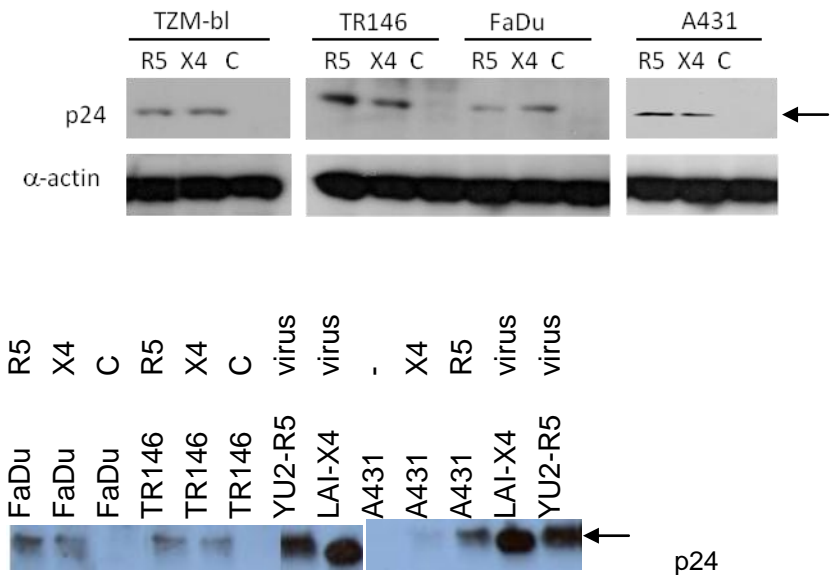


Figure 4-5: Western blot for p24 HIV-1 nucleocapsid using Gag monoclonal on epithelial virally exposed protein lysates. Top panel from Kohli *et al* 2012 (submitted manuscripts) performed by Arinder Kohli where TZM-bl is a positive control for virus binding. C represents untreated cell control. R5: YU2-R5, X4: LAI-X4. Bottom panel is own repeat. Antibody detects major band at 24kD indicated by arrow.

HIV-1 p24 capsid can be detected on or in the surface of FaDu, TR146 and A431 cells (Figure 4.4). Cells exposed to both viral tropisms tested positive for p24. Given that the stock concentrations of the viruses varied, a concerted effort was made to infect the epithelial cells with the same MOI across the viral subtypes. However, despite this, it is possible that the differences in intensity of p24 detection may be due to slight differences in virus MOI.

4.4 Discussion:

The objective of this chapter was to determine whether HIV-1 binds to oral and vaginal epithelial cell lines. To achieve this, two separate assays were developed to monitor HIV-1 presence on the surface of epithelium. The first assay detected surface HIV-1 gp120 on epithelial cell lines using two human monoclonal antibodies by flow cytometry and the second assay detected the presence of the capsid p24 by Western blot. The data conclusively demonstrates that HIV-1 R5 and X4 trophic viruses are able to directly bind oral and vaginal epithelial cells.

First, flow cytometry was utilised to detect HIV-1 bound to the surface of epithelial cells using two monoclonal antibodies: F425-B4e8 and F425-A1g8. F425-B4e8 was isolated from an HIV-1-infected individual (Bell et al., 2008) and recognizes the crown/tip of V3 variable loop on the gp120 subunit (via IleP₃₀₉ and ArgP₃₁₅). This region is critical for viral infectivity of permissive cells (Bell et al., 2008) and F425-B4e8 has been shown to be broadly neutralizing for HIV-1: of 40 primary HIV-1 isolates, F425-B4e8 neutralized half of clade B (n=16), one clade C (n=11) and one third of clade D (n=6) viruses (Pantophlet et al., 2007). The origin or epitope binding region of the second antibody, F425-A1g8, is unknown and no information was present on the NIH AIDS Reagent data sheet or in the PubMed database. However, its nomenclature implies that this antibody was probably isolated from the same study that isolated F425-B4e8 (Bell et al., 2008). F425-A1g8 is unlikely to be neutralizing since it is not reported in the literature.

Data with both antibodies suggest that R5 and X4 viruses bind to all epithelial cell types (Figs 4.2 and 4.3). However, using F425-B4e8, more HIV-YU2 (R5-trophic) virus (~60%) bound to the susceptible TZM-bl cells (positive control) compared with HIV- LAI (X4-trophic) (~23%), despite there being higher surface expression of CXCR4 on the surface of these cells (Chapter 3). This large difference in R5 versus X4 binding was not observed using the F425-A1g8 monoclonal antibody (both bound ~60%). This suggests that the epitopes detected in the V3 loop region of gp120 by F425-B4e8 and F425-A1g8 are different, with the F425-B4e8 epitope being partially obscured when X4 binds to CD4/CXCR4 on TZM-bl cells and the F425-A1g8 epitope remaining available for binding. Importantly, given that this difference in R5 and X4 binding with F425-B4e8 is not observed with TR146, FaDu or A431 cells, this indicates that X4 (and probably R5 virus) binding to epithelial cells utilises gp120 regions that are different to those regions used to bind permissive cells.

The F425-B4e8 antibody (and F425-A1g8) used in flow cytometry has a significant amount of background staining that is non-specific to HIV gp120 on the epithelial cell lines as shown in Figure 4.1 in the second column from the left where cells are primary and secondary stained for gp120 with no HIV. Given that the antibody

showed high background staining in flow cytometry and by ELISA (data not shown), the data must be interpreted with caution. Perhaps future experiments could explore further methods of blocking prior to antibody staining such as using purified human polyclonal Ig or inactivated human serum. Furthermore, additional antibodies could be tested that could provide a reduced level of non-specific fluorescence. Antibodies not directed against the V3 loop of gp120 should be tested. Only using antibodies directed to this epitope, that is masked until after CD4 binding (Checkley et al., 2011), may only allow the detection of a gp120 subset in a specific conformation open conformation. As far as we are aware, the conformation of gp120 in complex with all alternate receptors is not known and we cannot be certain that we are detecting all the gp120 on the epithelial surface. Furthermore, gp120 and p24 can be shed by infected cells (to the viral supernatant) and may not be part of whole virus particles, therefore some of what we observe may be just these two proteins (not associated with infectious virions). Ideally, electron microscopy would be used to visualise virus particles in contact with epithelium. This would also allow one to distinguish internalised versus external particles.

The flow cytometry R5 and X4 virus binding data was supported by Western blot data demonstrating detection of the p24 nucleocapsid after exposure of HIV-1 to epithelial cells. However, unlike the flow cytometry data, Western blots are not quantitative and only provide a qualitative measure of R5 and X4 binding to epithelial cells i.e. differences in binding capacity are not reliably revealed. Irrespective, both qualitative (Western) and quantitative (flow cytometry) approaches demonstrate that R5 and X4 virus bind oral and vaginal epithelial cells.

The quantitative flow cytometry data suggest that R5 and X4 virus are able to bind pharyngeal (FaDu) cells (~30%) better than buccal (TR146) and vaginal (A431) cells (~5%) (Fig 4.2 and 4.3). This was an unexpected finding but the trypsin digest experiments potentially provide some explanation into the nature of HIV-1 binding to the different epithelial cell types (Fig. 4.4). First, that HIV-1 binds to epithelial cells in both a trypsin-sensitive and -insensitive manner, suggesting that virus binding is mediated by protein and non-protein moieties. Second, the predominant mechanism of R5 binding is protein-mediated (since binding is reduced by at least 75% for all epithelial cell types and abolished in FaDu cells after trypsin treatment) and the predominant mechanism of X4 binding is non-protein mediated (since binding is generally more resistant to trypsin treatment, except for FaDu cells). Indeed, the majority of X4 binding to buccal and vaginal cells appears to be mediated via non-protein moieties or is internalized and detected. Fourth, intriguingly, that the mechanism of binding of HIV-1 to buccal and vaginal cells appears more similar than that between buccal and pharyngeal cells (both oral), in terms of trypsin sensitivity for R5 virus and trypsin resistance for X4 virus. One

could argue that if R5 and X4 virus utilised the same surface moieties to bind buccal, pharyngeal and vaginal epithelial cells, then an equal level of sensitivity to trypsin treatment should have been observed. Since this was not the case, it is possible that HIV-1 R5 and X4 utilise predominantly non-protein moieties (such as GalCer or carbohydrate/lectin-based molecules (Alfsen and Bomsel, 2002)) to bind to pharyngeal cells but protein moieties (such as HSPGs (Alfsen et al., 2005) or ICAM-1 (Paquette et al., 1998)) to bind to buccal and vaginal epithelial cells. However, this hypothesis would need to be fully explored by receptor blocking experiments using monoclonals against GalCer, HSPGs (or heparanitase to remove HSPGs) and ICAM-1, then exposing the epithelium to HIV, washing and then detecting gp120 by flow cytometry. This could aid in identifying the surface moiety responsible for HIV interaction with epithelial lines.

Given the low density of envelope spikes on the surface of HIV-1, perhaps the detection of gp120 data post trypsinisation could be noise or reflects a conformational change that is caused by trypsin treatment in either gp120 or another protein in the epithelial membrane.

A search of the literature indicates that our flow cytometry approach to evaluate HIV-1 binding to the surface of epithelium appears relatively unique. Therefore, our data must be put into context with similar studies investigating the interaction of epithelium with HIV-1 using other methods. Kage *et al.* (1998) showed by light microscopy that microparticles coated with gp120 were taken up by primary gingival epithelium, suggesting a binding and internalization mechanism. Our method on the other hand directly visualizes the virus particles using gp120 envelope protein as a marker on the epithelial surface since we do not permeabilize the cells prior to flow cytometry. Thus, the two studies show different processes. Other studies use p24 ELISA of protein extract samples (Saidi 2007, Alfsen 2005) in order to detect virus binding, but the ELISA method does not distinguish between surface viral binding or degradation of the virus after internalization and digestion. Finally, Nittayananta, *et al* (2009) used confocal microscopy of fluorescently labeled HIV-1 particles on differentiated oral epithelial models and observed that the majority of virus particles bound to the apical surface of the epithelial tissue with a few particles transcytosing, suggesting HIV-1 binding and potential transmigration across the epithelium. Although we did not utilize reconstituted multi-layer epithelial models, our approach detecting gp120 on epithelial monolayers also demonstrates evidence of HIV-1 binding to the surface of oral and vaginal epithelial cells.

4.5 Conclusion

The aim of this chapter was to determine whether HIV-1 binds to oral and vaginal epithelial lines. To achieve this we developed a flow cytometry assay with two separate monoclonal antibodies to detect surface bound gp120 and a Western blot assay detecting p24 capsid. Both approaches demonstrated direct (but low level) binding of HIV-1 R5 and X4 to oral and vaginal epithelial cell lines. Trypsin digest experiments revealed that the predominant binding mechanism of R5 virus to all epithelial cell types and for X4 virus to pharyngeal epithelial cells is via protein moieties, whereas the predominant binding mechanism of X4 virus to buccal (TR146) and vaginal (A431) epithelial cells is via non-protein moieties. However, it should be noted that HIV-1 binding to epithelial cells may be via protein moieties that are trypsin-insensitive. The direct interaction between HIV-1 and epithelial cells raises the possibility that HIV-1 may be internalized and its genome integrated into the epithelial DNA to support productive viral infection. This was addressed in Chapter 5.

Chapter 5 HIV integration

5.1 Introduction

In chapter 3, we demonstrated that oral and vaginal epithelial cell lines do not or very poorly express CD4, CCR5 and CXCR4 on the cell surface but, rather, they express non-canonical HIV-1 receptors including GalCer and HSPG's. Chapter 4 demonstrated that R5 and X4 virus are capable of binding to both epithelial cell types. Therefore, we hypothesised that HIV-1 binding may result in viral uptake and integration of the viral genome into epithelial cell DNA, potentially establishing a productive viral infection. This chapter investigates whether R5 and X4 virus integrates into oral and vaginal epithelial DNA.

HIV-1 integration was chosen as the step to monitor in order to monitor whether epithelial cells are productively infected. When HIV-1 infects a cell, the virus must enter the cell either by fusing with the plasma membrane through interaction with CD4 and its co-receptors (reviewed in (Checkley et al., 2011) and (Turner and Summers, 1999)) or alternate non-canonical receptors (Alfsen et al., 2005; Vacharaksa et al., 2008), or by internalisation via the endocytic pathway (Miyauchi et al., 2009). The virus then uncoats and a viral reverse transcriptase transcribes the plus stranded RNA to double stranded DNA in multiple steps classified as early, intermediate and late events that can be monitored by PCR (Mbisa et al., 2009). Not every reverse transcription event leads to integration and dead end products of linear or circular viral DNAs 1-LTR and 2-LTR-circle products may be created (Mbisa et al 2009). Integration occurs at the end of the early phase of HIV-1 infection (Turner and Summers, 1999) and once a host cell has an integrated copy of the viral genome its DNA coding material has been altered permanently. This final step of viral genome integration into the epithelial cell DNA was, therefore, chosen to assess HIV-1 infection.

In the literature, many published papers use the method described by Brussel *et al.* (Brussel and Sonigo, 2003, 2004) to show HIV-1 integration in human cells (Garvey et al., 2008; Goffinet et al., 2007; Nishimura et al., 2009; Vacharaksa et al., 2008). This is a two-step nested PCR method where repetitive *Alu* sequences in the human genome are utilized to enrich for integrated HIV-1 LTR sequences in cellular DNA, followed by an HIV-1 specific second round of amplification, yielding a measure of HIV-1 integration (Brussel and Sonigo, 2003).

The only directly relevant study to our own work was performed by Vacharaksa *et al* (2008) who used the Brussel *et al* method (2003) to show that HIV-1 may directly integrate into the genome of the OKF6/TERT-2 (floor of mouth) oral epithelial cell line without establishing a productive infection. However, it is hard to understand why a

provirus that is efficiently integrated in the cellular chromosome is unable to drive viral gene expression and subsequently a productive infection. It appears that rather than DNase-treating their virus stocks to remove plasmid/non-integrated HIV-1 DNA contamination (as in Brussel et al 2003, 2004), Vacharaksa et al (2008) instead performed nuclear extraction of cellular DNA to eliminate possible contamination from virus producer cells and plasmid. To our knowledge, this is the only study to use this method to eliminate viral DNA contamination. Using this approach, Vacharaksa et al (2008) showed that after 3-72 h incubation of OKF6/TERT-2 cells with the virus, integrated HIV-1 LTR was detected by visualising an amplicon using agarose gel electrophoresis. Using the same protocol, the authors also showed that viral integration was stable for three subcultures of the OKF6/TERT-2 cells (Vacharaksa et al 2008).

Other studies have claimed productive viral infection of epithelial cells by reporting p24 accumulation in epithelial culture medium (Han et al., 2000; Liu et al., 2003; Pang et al., 2000) or by demonstrating that trypsin-treated epithelial cells can transfer infectious virus to permissive cells (Giacaman et al., 2008; Vacharaksa et al., 2008). However, in Chapter 4 we found that trypsin treatment does not remove all virus from the epithelial cell surface and that p24 can be detected on the epithelial cell surface after viral exposure. Therefore, the claims of the above studies may not be the result of productive viral infection from within the epithelial cell but simply HIV-1 that remains bound on the epithelial surface during initial exposure.

For this study, we first utilised the Brussel method (2003, 2004) to investigate HIV-1 integration into epithelial cell DNA. However, this did not yield perfectly additive fluorescence curves for the two-step *Alu*-LTR HIV-1 PCR with increasing integrated copies at increasing numbers of infected cells. Therefore, we modified and optimized the protocol, with an unchanged first round enrichment followed by second amplification step performed using reagents and cycling times for qPCR. However, the final amplicon products were detected by agarose gel electrophoresis as in Vacharaksa *et al.* (2008) (Chapter 2, section 2.7.1). Irrespective of our optimising protocols, we still found that this two-step *Alu*-LTR PCR method to determine HIV-1 integration was liable to contamination by plasmid/non-integrated viral DNA. Therefore, the Brussel method was abandoned and replaced with a more reproducible and specific primer-probe qPCR design method (Chapter 2, section 2.7.2), which was adopted for this study. We also tried to produce infectious HIV from the epithelial lines but despite 5-10% transfection efficiency with GFP plasmid of A431 and TR146, we were unable to make any measurable titre virus using an infectious virus vector system (data not shown) which is consistent with the cells not being able to harbour productive infection.

The main aim of this chapter was to determine whether positive integration occurred in buccal oral epithelial cells (TR146) and to verify this data in oro-pharyngeal (FaDu) and vaginal (A431) epithelial cells. However, after extensive investigation we found no evidence of HIV-1 integration in oral, oro-pharyngeal or vaginal epithelial lines.

5.2 Methods

Methods are as detailed in Chapter 2, section 2.7.1, for the *Alu*-LTR-based nested-PCR assay. Briefly, HIV-1 (either DNase treated virus stocks or not) exposed epithelial cells were lysed for DNA and were used in first round PCR to amplify *Alu*-LTR sequences. Then an aliquot of these “enriched” products was used for the second round of amplification within the HIV-LTR.

Methods for the definitive primer-probe assay for HIV-1 integration are detailed in Chapter 2, section 2.7.2. Briefly, virus stocks were DNase-treated prior to infection of epithelial cells and compared with matched heat-inactivated viruses from the same stocks. Using a primer-probe assay, epithelial cell lines were tested after 48 h incubation with HIV-1 R5 and X4 to allow sufficient time for infection to occur. After thorough washing, cellular DNA samples were isolated and DpnI treated to eliminate plasmid DNA contamination prior to PCR amplification with a specific primer probe design.

Table 5-1: Recommendations for optimising qPCR methods

1. Use previously described primers for a given gene, using established conditions, but this may need optimization.
2. Use an on-line tool for primer design such as OligoArchitect™ (Sigma-aldrich). Contact Sigma-aldrich (UK specialist Dr Tania Nolan) and/or attend a workshop or course on primer design and qPCR. Refer to Nolan et al (2006).
3. BLAST search the primers to ensure they will not amplify other undesired products.
4. Avoid runs of nucleotides of more than 4 bp long.
5. Design primers 19-22bp in length that are approximately equal in GC versus AT content. Using a 3' GC-clamp (3-4 G and/or Cs) will promote primer binding to the template.
6. Be aware of intron-exon boundaries and splice variants of amplified gene of interest.
7. Verify that the primers amplify the predicted length amplicon around 100-200bp (not several bands) in a conventional PCR reaction. The ideal size is about 150 bp.
8. Choose an appropriate detection chemistry for your purpose: Sybr green can be easy since it is incorporated into the amplification product (and the assays are inexpensive) but a primer-probe design can be more specific to your template of interest.
9. If no amplicon is detected, reduce or increase the annealing temperature, vary the elongation temperature or the fluorescence acquisition read temperature. Also optimize $MgCl_2$ concentrations and primer concentrations
10. If many peaks are observed for fluorescence alter Sybr cycling conditions..
11. Use known amount of template in a duplicate or triplicate dilution series to verify that the C_t (at log phase) increases with greater DNA concentration (especially important if trying to quantify gene of interest).
12. Ensure the template is of high quality (e.g. not degraded).
13. The goal is to have a PCR reaction where the baseline is flat, the threshold is set in the log portion of the amplification curve, and that the curves of increasing DNA concentration are parallel in log phase. Plot C_t versus quantity and a linear curve should be obtained of the form $y = mx + b$. Ensure the slope of curve (m) is close to -3 to -3.9 and that r^2 is close to 1.

5.3 Results

5.3.1 Integrated HIV-1 detection by *Alu*-LTR-based nested-PCR assay

Brussel et al (2003) described a method to detect integrated HIV-1 in human cells whereby two outward facing *Alu*-sequence specific primers are included with an HIV-1 LTR DNA specific primer (Figure 5.1a, R region) in the first round PCR for twelve cycles. *Alu* sequences are widespread throughout the human genome with an average distance between elements of approximately 4 kb (Britten et al., 1988). Since the viral integration event can occur anywhere in the epithelial genome, it is likely to be in close proximity to an *Alu* sequence, hence these repetitive sequences are used to enrich for HIV-1 integration into human DNA in the first round of PCR. The second PCR step is nested, using HIV-1-specific sequences with primers targeting Lambda T and another HIV-1 LTR specific primer (Figure 5.1b, U5 region) for 24 cycles. This generates a small product that can be used to determine HIV-1 integration (Brussel and Sonigo, 2003, 2004).

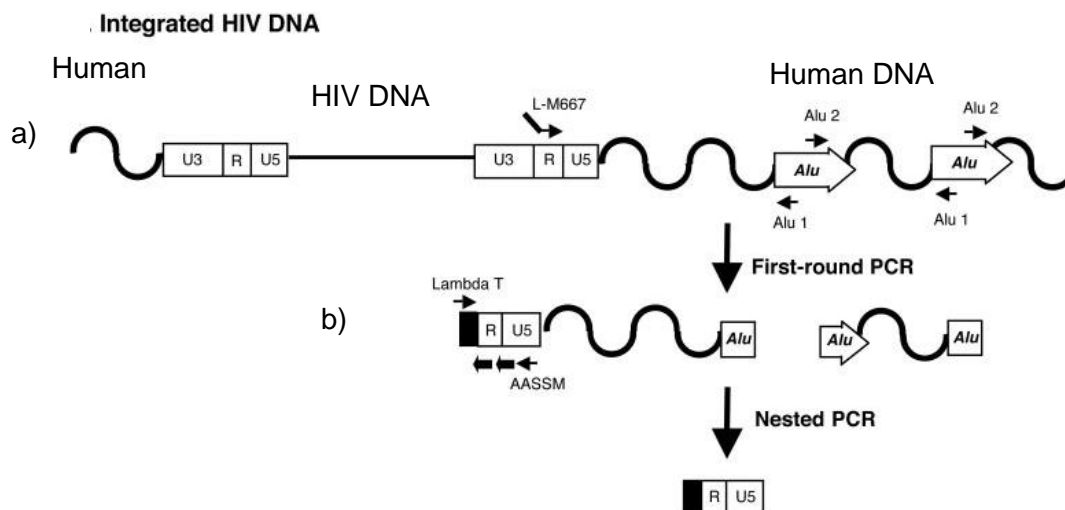


Figure 5-1: Integrated HIV-1 detection by *Alu*-LTR-based on a two step nested-PCR assay design (taken from Brussel et al 2004). Host DNA is enriched using two outward facing primers complimentary to human *Alu* sequences in conjunction with HIV-1 LTR specific primer, L-M667. The second round is a nested PCR that specifically amplifies HIV-1 specific sequences from Lambda T to U5 within the LTR region. The limit of detection is 10 copies per sample (Brussel et al 2003).

This assay was initially utilized to detect integrated HIV-1 in oral and vaginal epithelial cell lines (Figure 5.2). TZM-bl cells were not used for this assay since these cells test positive for integrated HIV-1 (our observation in Figure 5.3b). This is because they were constructed to express β -galactosidase under the control of the HIV-1 LTR (Wu X. et al., unpublished data (Derdeyn et al., 2000)). Therefore, PM-1 cells were used as a positive control for infection. Initially, virus stocks were not DNase-treated since we generated stocks by propagating the virus through susceptible suspension cells.

Mistakenly, we assumed that the ability of virus producer cells to contaminate the PCR with their integrated HIV-1 DNA would not be an issue after extensive washing of the virally exposed epithelial cells prior to DNA extraction. Figure 5.2 shows a representative result with HIV-1 exposed epithelial lines from this experiment. The TR146 and FaDu cells appear to have integrated copies of HIV-1 when exposed to R5 (YU2) and X4 (LAI) virus (middle panel), similar to the susceptible PM1 control cells (left panel).

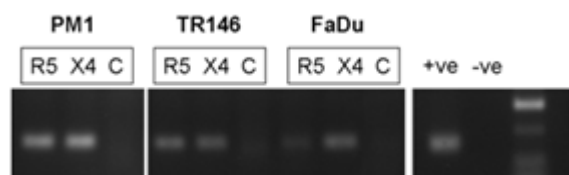


Figure 5-2: 2nd round integrated PCR detection for epithelial lines exposed to HIV-1 using the Alu-LTR primers (Brussel et al 2003, 2004). R5 is YU2, X4 is LAI. C refers to an untreated cell control. +ve: 8E5/LAV CEM-derived human T-cells, containing a single integrated copy of the HIV genome per cell. -ve: no-template control. Ladder, far right. From Kohli et al 2012, submitted.

Given that these initial studies appeared to demonstrate that HIV-1 was able to integrate into the epithelial cell DNA, we attempted to confirm and qualify the data further. First, we investigated whether viral integration was dependent on the presence of the envelope protein by using an envelope-deficient virus (HIV-gpt) or this same virus complemented with gp160 of YU2 (Chapter 2, section 2.1.3.1). Second, we determined whether viral integration could be inhibited by reverse transcriptase (AZT) or integrase inhibitor (118-D-24 or 4-[3-(azidomethyl)phenyl]-2-hydroxy-4-oxo-2-butenic acid). Both drugs should prevent HIV-1 integration and were used as negative controls. Finally, we investigated whether incubation of epithelial cells with small amounts of plasmid HIV-1 molecular clone DNA resulted in detection of integration. The data from these additional studies demonstrated fundamental flaws in the reliability and consistency of this assay system, which can be observed when analysing the *Alu*-LTR 2nd round PCR amplification data for A431, FaDu and TR146 cells in Figure 5.3.

For example, in Figure 5.3A and B, A431 YU2 (R5) incubated with AZT treatment (lane 5) or integrase inhibitor (lane 8) tested positive for viral integration while LAI (X4) tested negative (lanes 6 and 9 respectively). Similar confusing data is shown for FaDu cells, YU2-AZT tests positive (A, lane 16) and LAI incubated with integrase inhibitor tests positive for integration (B, lane 20). For TR146 the data is equally confounding: they test positive for integration with YU2 in the presence of AZT (Fig. 5.3B, lane 31). For all these, one would expect no integration events in the presence of AZT or integrase inhibitor if integration was due to virus infection of the cells. In addition, data were inconsistent as demonstrated by positive integration events for LAI virus in TR146 and

FaDu cells in Fig 5.2 but no detectable integration for LAI in either cell type in Fig 5.3 (lanes 15 and 30). Finally, the addition of 1 ng viral plasmid DNA alone, followed by extensive washing, also resulted in erroneous detection of positive integration events (contamination) in TR146, FaDu and A431 cells (lanes 10, 21, 34). Together, these experiments demonstrate that the positive integration events we observed are very likely due to contaminating plasmid rather than viable virus infecting and integrating its genome into the epithelial DNA. We determined that the reason for this was the lack of DNase treatment of our original virus stocks. Finally, it should be noted that the use of TZM-bl cells in this experiment as a positive control was inappropriate since, due to their construction, they always test positive for integrated HIV-1, an error identified post-experiment (see untreated, lane 26).

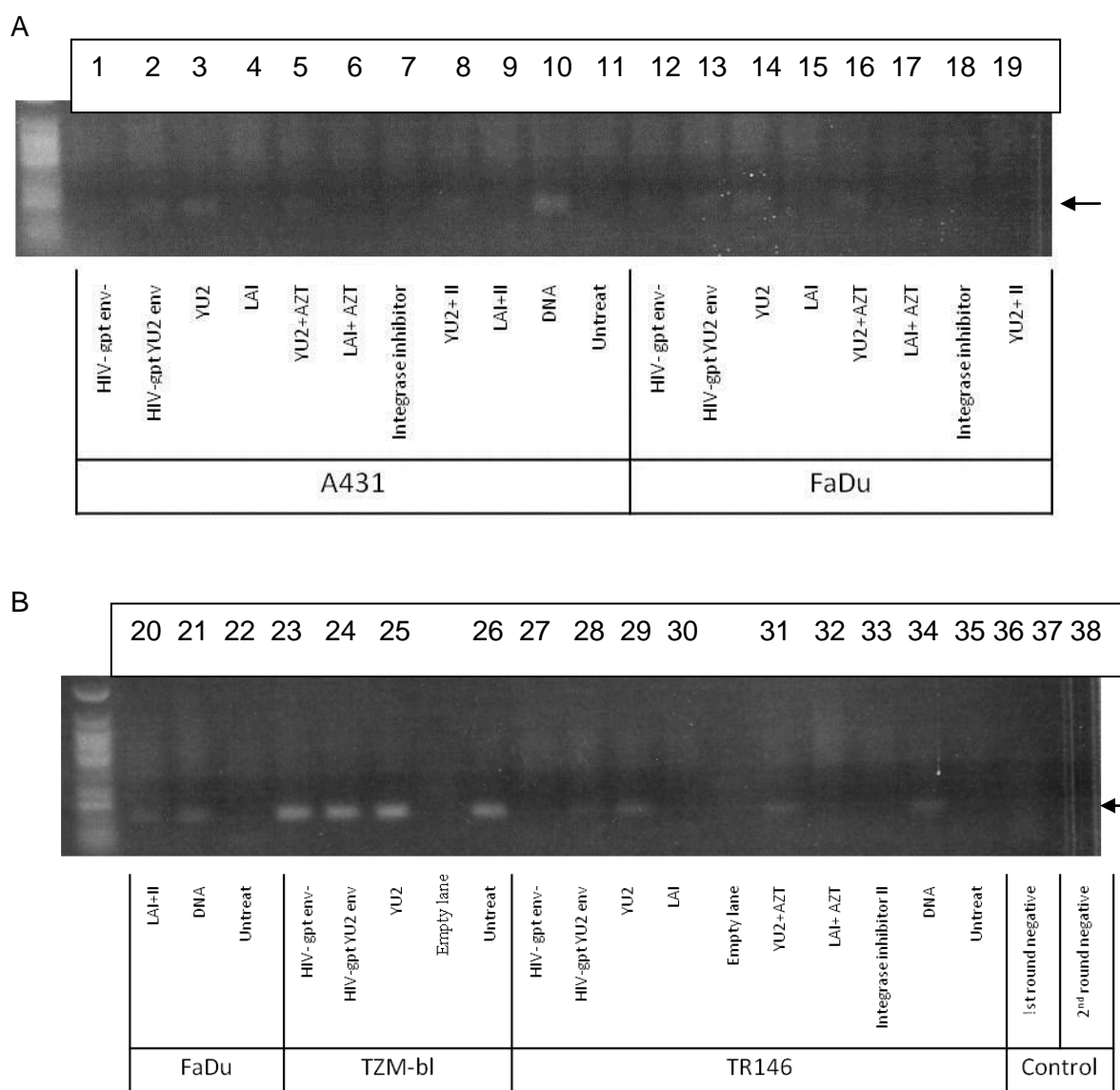


Figure 5-3: Detection of integrated HIV-1 DNA in TR146, FaDu and A431 epithelial cells using *Alu*-LTR PCR. Cells were grown in monolayers, exposed to the described conditions and genomic DNA isolated. A two-step nested PCR was performed using primers and protocols described by Brussel et al (2003, 2004) and the second round of PCR amplification is shown. HIV-gpt with no gp160, HIV-gpt complemented HIV-gpt with YU2 envelope, YU2 (R5) HIV-1, LAI (X4) HIV-1, pre-incubation with AZT followed by YU2, pre-incubation with AZT followed by LAI, pre-incubation with integrase inhibitor, pre-incubation with integrase inhibitor and YU2, pre-incubation with integrase inhibitor and LAI, no virus but only 1 ng plasmid DNA.

Given the apparent importance of DNase treatment for assessing integration of HIV-1 DNA, we decided to DNase treat the virus stocks prior to experimentation to determine whether this treatment would be sufficient to remove the false positive data and to provide reproducibility to the Brussel assay (Brussel and Sonigo, 2003). On this occasion, we used susceptible PM-1 cells rather than TZM-bl cells as a positive control. Additionally, we only performed the experiment with LAI (X4) (as a representative virus) since large quantities of high-titre YU2 (R5) stocks were difficult to produce. PM-1 cells

were exposed to (i) LAI (X4) with and without DNase treatment and (ii) different concentrations of plasmid DNA (1 ng and 1 µg) with and without DNase treatment, and integration events determined. Figure 5.4 shows the 2nd round PCR amplification data for the *Alu*-LTR assay (Brussel et al 2003, 2004). PM-1 cells incubated with LAI (X4) after two types of DNase treatment (DNase with and without its own buffer) did not appear to have integrated HIV-1 DNA (lanes 1 and 2), whereas untreated (no DNase) virally exposed PM-1 cells tested positive (lanes 3). The former data set was not expected as the DNase-treated LAI (X4), being a viable whole virus, should integrate into the dual trophic permissive PM-1 cells. Notably, PM-1 cells exposed to 1 ng or 1 µg of plasmid DNA tested positive for integration only when not DNase-treated (lanes 4 and 5 versus 6). It should be noted that a very faint amplification product was observed for 1 µg of plasmid DNA after DNase treatment (lane 7) but this probably reflects the presence of insufficient amount of DNase to remove all the plasmid. Thus, these data demonstrate that the Brussel assay as we carried it out appears exquisitely sensitive to plasmid contamination and that DNase treatment is essential to prevent false positive integration events. However, it also highlights a lack of confidence in this assay since the DNase-treated LAI (X4) should integrate into the permissive PM-1 cells.

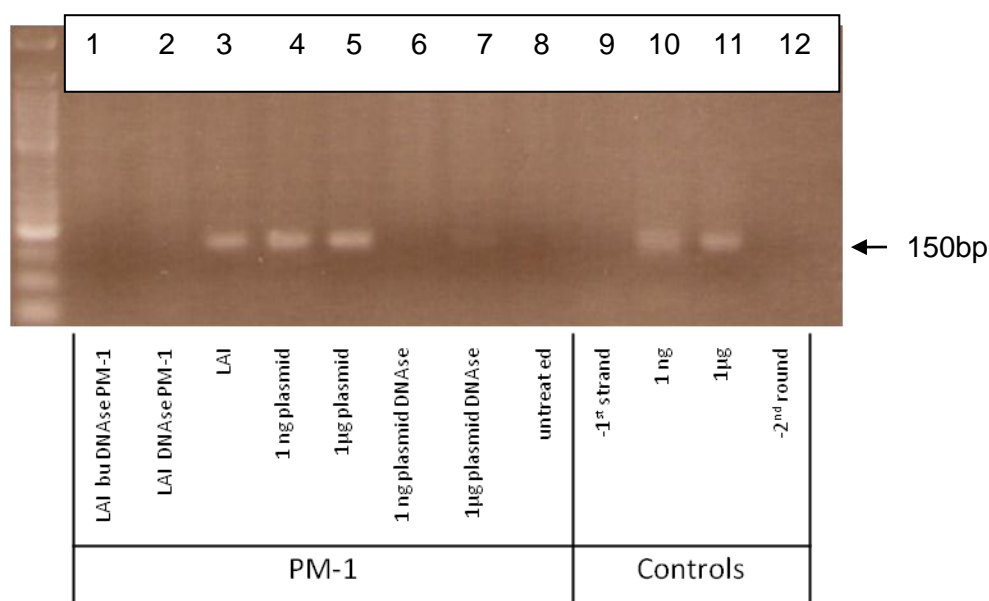


Figure 5-4: Second round integrated *Alu*-LTR PCR for HIV-1 integration with PM-1 cells incubated under various conditions. For brief methods see legend to Figure 5.3. LAI X4 virus DNase treated with or without its own buffer, untreated LAI, PM-1 cells exposed to 1 ng or 1 µg plasmid DNA with and without DNase treatment, and untreated cells. Also included in controls are 1st round PCR no template control, 1 ng or 1 µg plasmid DNA, and a no template control for the 2nd round PCR.

To confirm our hypothesis, we additionally analysed the amplicon product amplified from the first round of PCR (Figure 5.5.). It was clear that the first round enriching step

was amplifying plasmid DNA at both the 1 ng level (showing amplification of a specific 150 bp LTR region) -second lane from right and more so at the 1 µg level, where it appears the whole circular plasmid has been amplified (top of gel-far right lane). In summary, these investigations indicated that the Brussel assay was susceptible to providing false positive data for viral integration as a result of plasmid contamination that could not confidently be resolved solely by DNase treatment. Therefore, we abandoned this PCR assay in search of a more precise and reliable assay based on quantitative PCR.

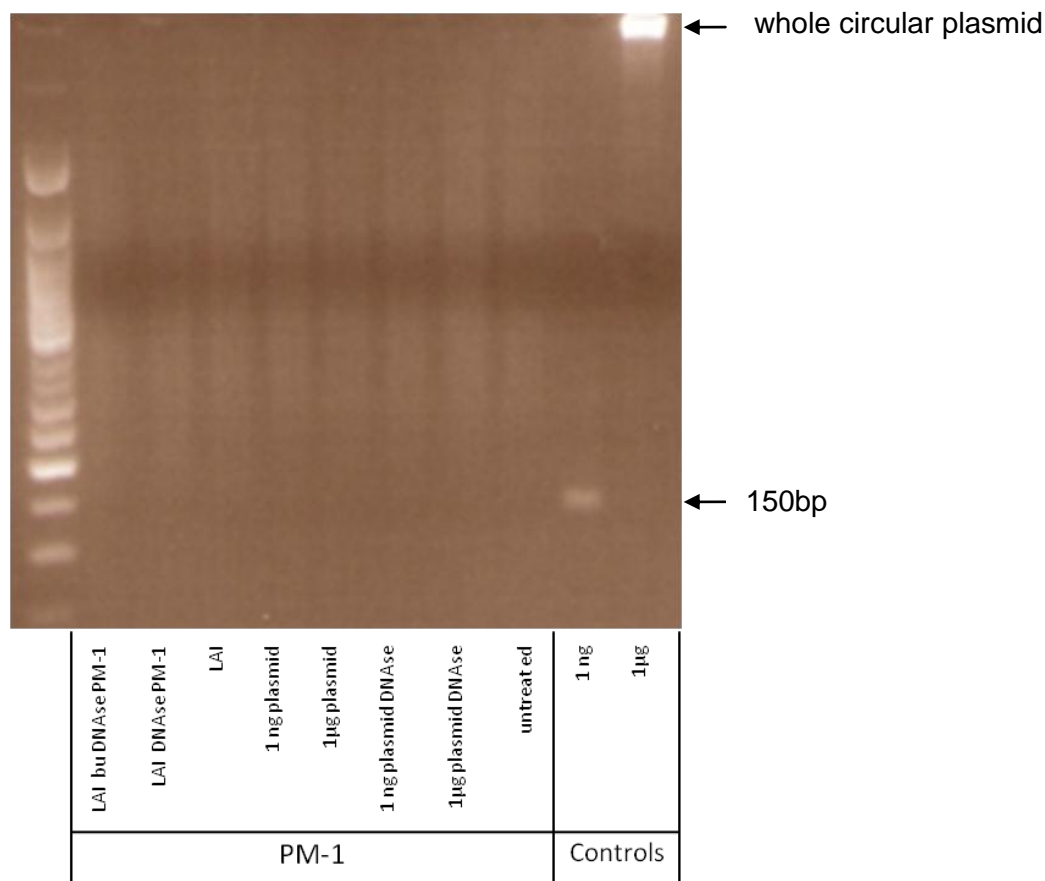


Figure 5-5: First round *Alu*-LTR PCR of PM-1 samples. For brief methods see legend to Figure 5.3. LAI X4 virus DNase treated with or without its own buffer, untreated LAI, PM-1 cells exposed to 1 ng or 1 µg plasmid DNA with and without DNase treatment, and untreated cells. Controls are 1 ng or 1 µg plasmid DNA.

5.3.2 Primer-probe assay for HIV-1 integration

The assay described by Brussel et al (2003, 2004), which has been used in many published papers including Vacharaksa et al (2008), proved to be unreliable in our hands. Therefore, we searched for a different approach to investigate HIV-1 integration into epithelial cell DNA and subsequently found an elegant protocol described by Mbisa *et al* (2009) using qPCR. In this protocol, the forward primer binds to the U5 region of the

HIV-1 LTR, the reverse primer binds to the *Alu* repeat sequence in the host genome, and the internal FAM-TAMRA labeled probe binds in between these regions (but also binds the U5 region of the HIV-1 LTR) (Figure 5.6). The specific utilisation of the U5 specific FAM-TAMRA probe also adds specificity to qPCR assay. During PCR, this assay amplifies the majority of the HIV-1 LTR together with some host genomic material, and a PCR product can only be amplified if the HIV-1 LTR has integrated into the host genome.

In qPCR the DNA polymerase adds nucleotides to the primers and when it reaches the region bound by the probe in the annealing phase of the PCR cycle, the probe is displaced and degraded by the polymerase's exonuclease activity. Probe degradation leads to the separation of the fluorescent label (FAM) from its quencher (TAMRA), allowing the fluorescence to be emitted and recorded by the qPCR machine's detector (Mbisa et al 2009). Since the goal of the assay was solely to ascertain whether or not the epithelial lines comprised an integrated copy of HIV-1, it was deemed unnecessary to make the standard curve for quantitative assessment of the number of integrated copies as described in Mbisa (et al 2009).

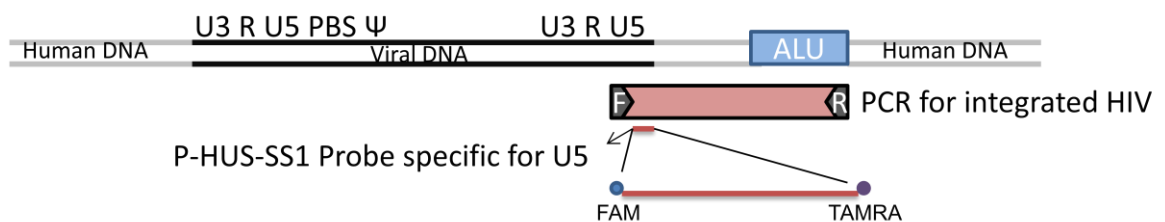


Figure 5-6: Primer-probe qPCR assay for detection of integrated HIV-1 DNA. Protocol adapted from Mbisa et al (2009). Reverse primer for *Alu* region was used in conjunction with an HIV-1 LTR, U5 specific primer, and U5 specific FAM-TAMRA probe.

Three major steps were taken to ensure that this assay was precise and would detect only positive integration events. First, to eliminate issues of contamination from plasmid or DNA from virus producer cells, virus stocks were treated with DNase prior to incubation with epithelial lines. Second, cells were carefully but extensively washed with PBS after viral incubation and the isolated genomic cellular DNAs were digested with DpnI, which only digests methylated plasmid DNA and not epithelial cellular DNA. Lastly, Mbisa et al (2009) describe the use of a heat-inactivated virus control to be assayed alongside the test samples. Thus, the same virus stock that was DNase treated and incubated with cells was heat-inactivated for 1 h for 60°C and amplified alongside the test sample. Heat-inactivated virus that is unable to gain entry to cells gives a quantitative measure of any remaining contaminating DNA in the system. In qPCR, a positive integration event will result in a lower C_t value (amplifies sooner) in the DNase-

treated virus sample when compared to the heat-inactivated virus. Absence of integration is demonstrated when the sample exposed to DNase-treated virus shows a lack of amplification or a higher C_t (amplifies later) value than the sample exposed to heat-inactivated virus.

Initially this qPCR assay was tested with PM-1 and C8166 cells, which are susceptible to R5 and X4 virus, respectively, and the raw data shown in Figure 5.7. Incubation of PM-1 cells (Figure 5.7a) with DNase-treated YU2-R5 virus (red) and plasmid DNA (blue) did not amplify an integrated product while PM-1 cells incubated with untreated heat-inactivated virus did amplify an integrated product (pale green). In a second graph from the same experiment, we investigated C8166 and PM-1 cells with X4 virus (Fig. 5.7b). The fluorescence plot shows a product for integrated HIV-1 with the DNase-treated virus in PM-1 (purple) and C8166 (blue) cells. For both cell types, the curve has a higher C_t value in the DNase-treated than the heat-inactivated virus treated cells (green). This confirms that this qPCR assay can reliably detect integrated X4 HIV in control cell lines (PM-1 and C8166). However, it also suggests that the PM-1 cells may not be the ideal cell type to assess HIV-1 R5 integration. Thus, in future assays we utilized the NP2-R5 cell line (that expresses CCR5 under drug selection) as a positive control in assessments of HIV-1 R5 integration.

Once the assay was established, we used the *Alu*-LTR primer-probe assay to verify whether HIV-1 YU2-R5 and LAI-X4 can integrate into TR146, FaDu and A431 epithelial cell lines after 48 h of incubation. Initial experiments indicated that no integration of DNase-treated HIV-1 was observed in the epithelial lines at an MOI of 1 (data not shown). We then decided to increase the MOI (7.6) in order to test whether this would allow detection of viral integration into the epithelial genome. We initially performed this experiment with the YU2-R5 virus. Figure 5.8 shows the fluorescent traces for epithelial cell lines incubated with DNase-treated YU2-R5, heat inactivated YU2-R5, and a combination of DNase-treated and heat-inactivated YU2-R5 (the latter being an additional control that we expected to have a reduced signal versus the two former conditions). It is clear that A431 (Fig 5.8a), FaDu (Fig 5.8b) and TR146 (Fig 5.8c) cells exposed to DNase-treated and heat-inactivated viruses do not amplify the integrated HIV-1 product. On the other hand, the control NP2-R5 susceptible cells do contain integrated HIV-1 because the DNase-treated virus amplifies at approximately 30 cycles (Fig 5.8d, beige line). In conclusion, HIV-1 YU2-R5 does not appear to integrate into the host genome when epithelial cells are incubated with approximately eight virus particles per cell.

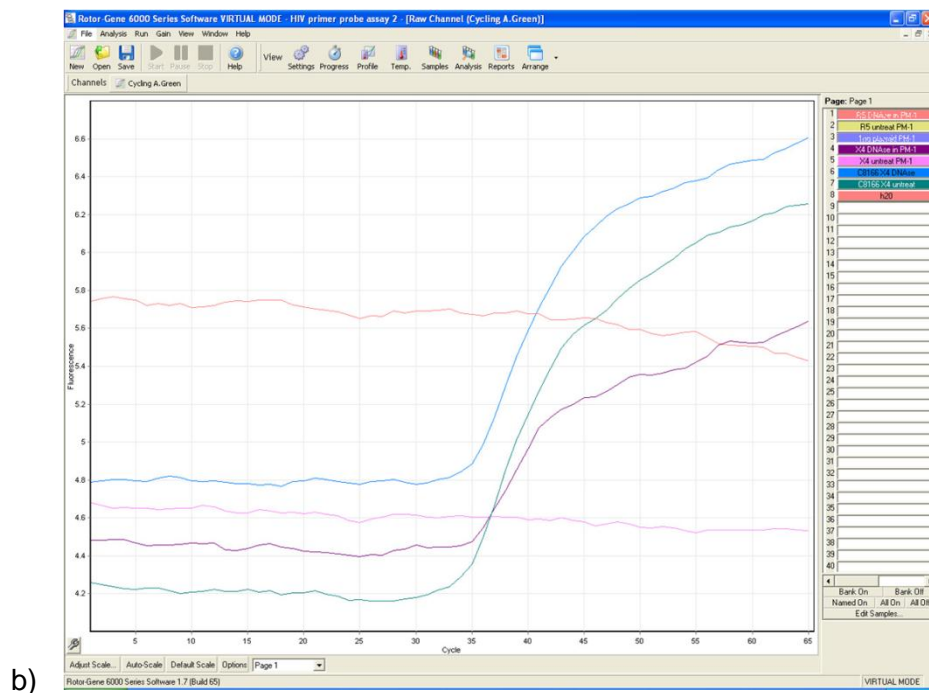
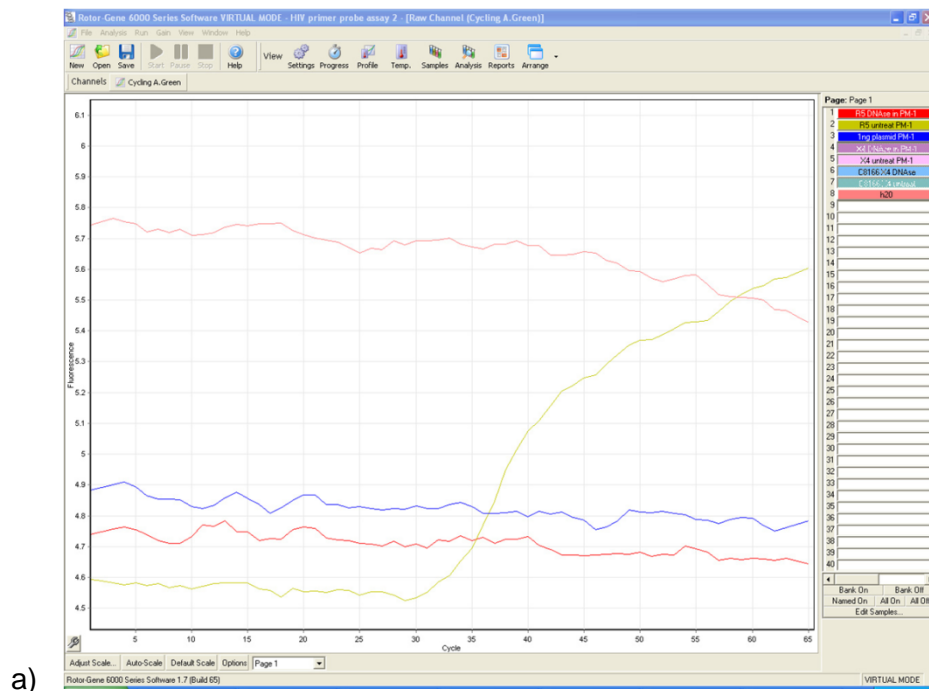


Figure 5-7 : Fluorescence traces for primer-probe *Alu*-LTR assay to detect HIV-1 integration. (a) PM-1 cells with YU2-R5 virus MOI 1 incubated for 48 h. Red line: DNase treated YU2 on PM-1 cells. Blue line: 1 ng plasmid DNA incubated on cells. Pale green: untreated and heat inactivated virus incubated with cells. Peach: no template control. (b) C8166 and PM-1 cells with LAI-X4 virus MOI 1 for 48 h. Pale blue line: C8166 cells with DNase treated X4. Teal line: C8166 untreated and heat inactivated X4. Purple line: PM-1 DNase treated X4. Pink line: untreated X4 and heat inactivated PM-1. Peach line: no template control.

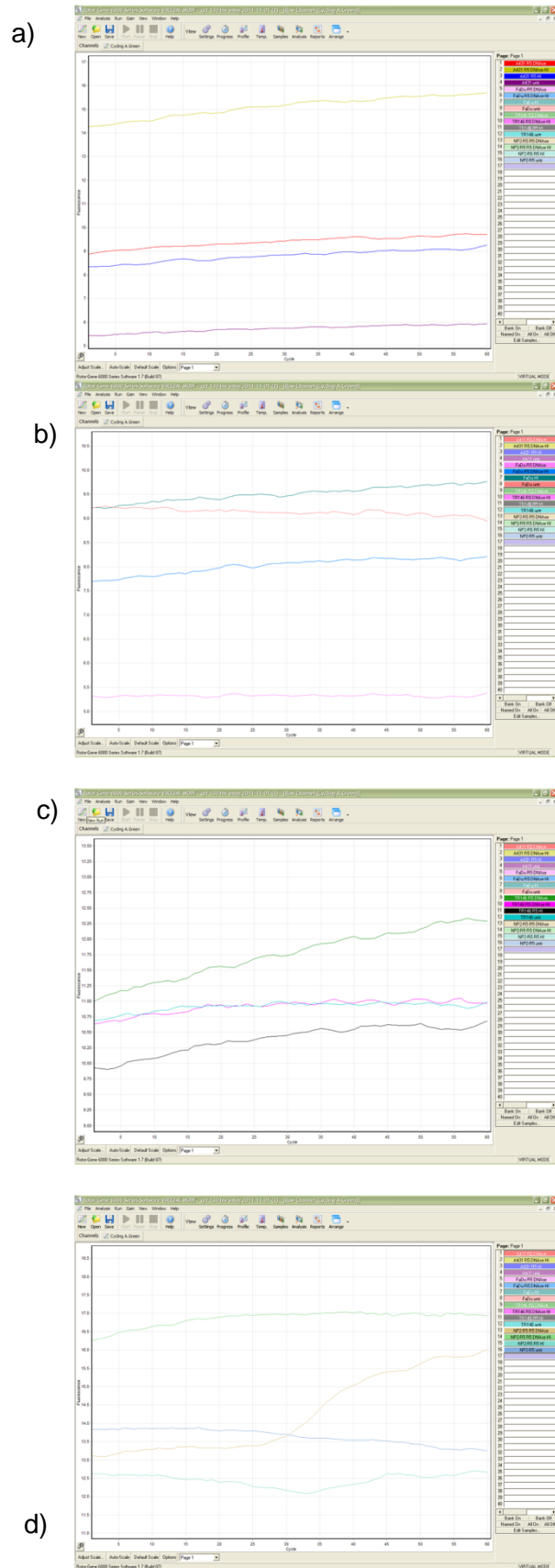


Figure 5-8: Fluorescence traces for primer-probe *Alu*-LTR assay to detect YU2 (R5) virus integration. Experiments were performed at an MOI of 7.6 and assayed after 48 h incubation. (a) A431 cells. Red line: DNase treated R5. Pale Green: DNase and heat inactivated R5. Blue line:

heat inactivated R5. Purple: untreated cell control. (b) FaDu cells. Pink line: DNase treated R5. Blue line: DNase and heat inactivated R5. Teal line: heat inactivated R5. Peach line: untreated cell control. (c) TR146 cells. Green line: DNase treated R5. Pink line: DNase and heat inactivated R5. Black line: heat inactivated R5. Pale blue line: untreated cell control. (d) NP2-R5 cells. Beige line: DNase treated R5. Pale green: DNase and heat inactivated R5. Sky blue: heat inactivated R5. Blue: untreated cell control.

We then proceeded to determine whether HIV-1 LAI-X4 was able to integrate into the genome of A431 and TR146 epithelial cells. TR146 cells were used as a representative cell line from the oral cavity (FaDu cells were not tested for this experiment). We observed a similar integration pattern with LAI-X4 as we did with YU2-R5. The experiment was performed at two MOIs; first at an MOI of 10 (Figure 5.9 for epithelial DNA PCR fluorescent traces and Table 5.1) and also at an MOI of 146 (Table 5.1: >100). Figure 5.9a shows that an integrated product is not detected from the DNA of A431 cells incubated with DNase-treated LAI-X4 (red line) while the heat-inactivated virus treated cellular DNA (blue), the control for contamination, does amplify. Identical data were obtained for TR146 cells, with only the control for DNA contamination (teal line: heat-inactivated virus) amplifying a product in the *Alu*-LTR primer-probe integrated HIV-1 PCR (Figure 5.9b). We next increased the MOI to >100 in order to further demonstrate the lack of viral integration even when virus is in excess. Again, no PCR product was amplified from DNA of TR146 or A431 epithelial cells exposed to DNase-treated virus (Table 5.1).

In summary, the *Alu*-LTR primer probe assay detects integrated DNA in control susceptible cells lines but does not support the hypothesis that epithelial lines exposed to HIV-1 become infected.

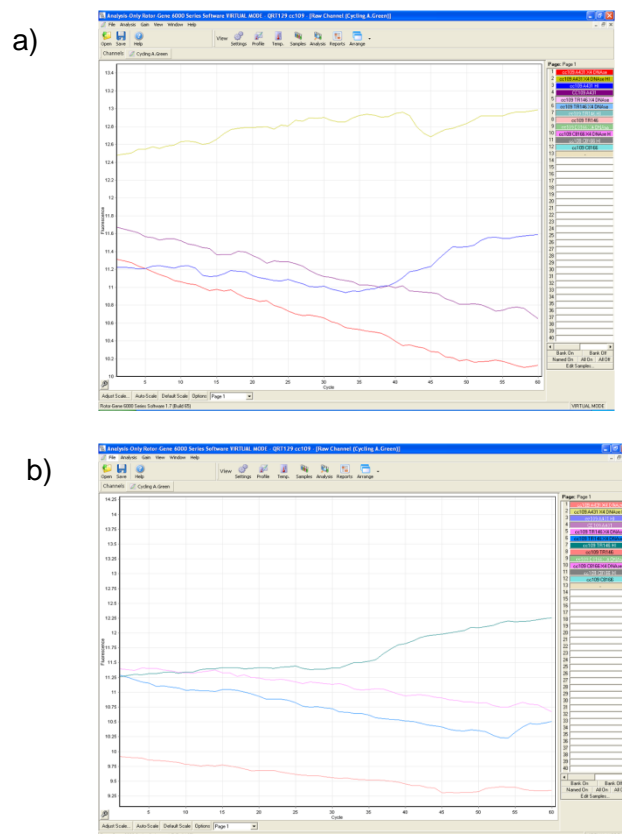


Figure 5-9: Fluorescence traces for primer-probe *Alu*-LTR assay to detect LAI (X4) virus integration. Experiments were performed at an MOI of 10 and assayed after 48 h incubation. (a) A431 cells. Red line: DNase treated X4. Pale green: DNase treated X4 and heat inactivated. Dark blue: heat inactivated virus. Purple line: untreated A431. (b) TR146 cells. Pink line: DNase treated virus. Teal line: heat inactivated virus. Blue line: DNase treated and heat inactivated. Peach line: untreated TR146.

Table 5-2: Detection of integrated HIV-1 in epithelial cell lines after 48h

Cell line	R5-YU2 (MOI:7.6)	X4-LAI (MOI:10)	X4-LAI (MOI >100)
FaDu	ND	Not included	ND
TR146	ND	ND	ND
A431	ND	ND	ND
C8166	n/a	+ (Ct = 32.7)	+ (Ct = 33.5)
NP2-R5	+ (Ct = 28.8)	n/a	n/a

ND, not detected (Ct value greater than 60)

n/a not included in experiment

5.4 Discussion

Viral integration into the host genome is an essential step for the subsequent production of infectious new virions. Therefore, the objective of this chapter was to

determine whether HIV-1 integrates into the genome of oral and vaginal epithelial cells. To achieve this, a two-step PCR and primer-probe qPCR assays were developed to monitor HIV-1 LTR integration using an *Alu*-LTR detection system. Using a sensitive and reliable primer-probe assay for integration we find no evidence that HIV integrates into the epithelial genome.

5.4.1 Two step *Alu*-LTR integrated HIV-1 DNA PCR

Using the *Alu*-LTR primers described by Brussel et al (2004) and an agarose gel readout system, integrated HIV-1 DNA appeared to be detected in the epithelial genome. However, when this PCR assay was tested rigorously it became apparent that the assay was susceptible to contamination by virus producer cell DNA and plasmid DNA. This was confirmed when DNase treatment of the virus abrogated the ability to detect integrated HIV-1 DNA. Furthermore, the addition of AZT (reverse transcriptase) and integrase inhibitors did not prevent detection of viral integration in the absence of DNase treatment. Therefore, we pursued a modified two-step nested PCR for the detection of integrated HIV-1 but similar results were obtained. Taken together, the data demonstrate that the two PCR based assay system was unreliable and was not showing true viral integration events.

Our data are in contrast to Vacharaksa et al (2008) who published data using the primers from this assay to show that oral epithelial OKF6/TERT-2 cells contained integrated HIV-1 DNA, which was stable for three generations. However, the method used by Vacharaksa differs from that of Brussel *et al* (2003, 2004). Firstly, the virus stocks were not treated with DNase prior to infection of the OKF6/TERT-2 cells and, secondly, the DNA nuclear extracts were used to avoid contamination when testing for viral integration (Vacharaksa *et al* 2008). They showed that heat-inactivated HIV-1 (70°C for 3 h), AZT and Colchicine (inhibits microtubule polymerization, cytoskeletal rearrangement and mitosis) pretreated cells did not amplify a 150 bp product for integrated HIV-1, thereby concluding that HIV-1 integrates into the epithelial genome. However, these data are suspect as the AZT and Colchicine treated cells and no template control all amplified a 'smear' approximately the same size as the 150 bp size product, which should not be present. It should be emphasised that nuclear extraction without DNase treatment of virus stocks may not have been sufficient to remove potential DNA contamination, and thus the use of nuclear extracts and heat-inactivated controls in the Vacharaksa *et al* (2008) study may not have been sufficient to prevent false positives for viral integration. The key experiment that these authors did not perform was to expose epithelial cells to different concentrations of viral plasmid, then nuclear extract epithelial cell DNA and perform the nested PCR to detect for viral integration. However, it should be noted that the differences between our study and that

of Vacharaksa et al (2008) might also reflect a difference in the properties of the cell lines used in this study (TR146, FaDu, A431) versus OKF6/TERT-2 cells. Thus, it is plausible that the OKF6/TERT-2 cells (floor of the mouth) are more susceptible to HIV-1 infection and display HIV-1 integration into their genome unlike TR146, FaDu, A431 cells.

Given the false positive data we obtained for HIV-1 integration using the two-step *Alu*-LTR PCR assay we concluded that it would be desirable to test for HIV-1 integration by a more specific and reproducible assay, namely the primer-probe *Alu*-LTR quantitative PCR assay.

5.4.2 Primer-probe *Alu*-LTR qPCR assay

We first established that the primer-probe *Alu*-LTR quantitative PCR assay (Mbisa et al 2009) was reliable, specific and sensitive. This was achieved by incorporating three aspects to the protocol: (i) DNase treatment of virus stocks to eliminate issues of contamination from plasmid or DNA from virus producer cells; (ii) digestion of epithelial DNA (post-infection) with DpnI, which only digests plasmid DNA and not epithelial DNA; and (iii) inclusion of a heat-inactivated virus control, which is unable to gain entry to cells and thus provides a quantitative measure of contaminating DNA in the system.

The primer probe *Alu*-LTR assay is straightforward and does not appear to be susceptible to contamination by plasmid DNA when the appropriate precautions are taken (see above). Fluorescence is emitted only when the probe is degraded by the exonuclease activity of the polymerase and, therefore, the assay is specific and reproducible. Using this assay we found no evidence for integration of R5 or X4 HIV-1 viruses after 48 h infection of oral (TR146, FaDu) or vaginal (A431) epithelial cells, even when testing high MOIs (up to 140 for X4 virus) that would be unlikely to occur during a natural exposure/infection episode. The only potential drawback of this assay could be the limit of detection. Mbisa et al used 100-500 ng of DNA per 50 μ l qPCR reaction while we used either 50 or 100 ng in a 10 μ l qPCR reaction, which is within the same order of magnitude for the reaction DNA concentration. However, if viral integration into the epithelial genome is a rare event and occurs at very low levels, it is possible that this assay may not be sensitive enough to detect the integration event.

5.5 Conclusion

The aim of this chapter was to determine whether HIV-1 was able to integrate into the genome of oral and vaginal epithelial cells. Initially a two-step nested *Alu*-LTR PCR designed by Brussel et al (2003, 2003) was used but this assay was found to be unreliable and vulnerable to contamination. To eliminate contamination, we used several

methods including DNase treatment of virus stocks prior to incubation with epithelium and DpnI treatment of extracted cellular DNA samples post-exposure to virus. By utilizing a more specific primer-probe qPCR assay to monitor HIV-1 integration (Mbisa et al 2009), we found no evidence for R5 or X4 viral integration into oral or vaginal epithelial cells, even when the virus titre was increased to >100 virus particles per epithelial cell. We conclude that although studied epithelial lines appear to express HIV-1 binding factors (Chapter 3) and HIV-1 is able to directly bind epithelium (Chapter 4), the virus is unable to integrate into the epithelial genome. Therefore, it is highly unlikely that epithelial cells become productively infected. However, this does not exclude the possibility that captured virus on the surface of epithelial cells could be transferred to other susceptible/permissive cells that may be present in the sub-mucosa. This hypothesis was examined in the next chapter (Chapter 6).

Chapter 6 Epithelial transfer of infectious virus to susceptible reporter cells

6.1 Introduction

In previous chapters HIV-1 was shown to bind to TR146, FaDu and A431 epithelial lines (Chapter 4) but failed to integrate into the epithelial genome (Chapter 5). Thus, the question arose as to whether HIV-1 remained infectious post-capture by epithelium and in a conformation that was transmissible to susceptible cells, permitting infection. Therefore, the aim of this chapter was to determine whether HIV-1 exposed oral, oro-pharyngeal and vaginal epithelial cells were able to transfer infectious virus to susceptible TZM-bl reporter cells.

Many studies have described the ability of oral and vaginal epithelium to transfer HIV-1 to susceptible, CD4 expressing, cell types (Asin et al., 2004; Asin et al., 2003; Bobardt et al., 2007; Bomsel, 1997; Carreno et al., 2002; Giacaman et al., 2008; Liu et al., 2003; Maher et al., 2005; Vacharaksa et al., 2008; Wu et al., 2003). For oral and pharyngeal epithelial sites only four studies appear to have been published. Liu *et al* (2003) incubated normal human oral keratinocytes (NHOK) with HIV-1 Ba-L and NL4.3, washed and then incubated them overnight with peripheral blood lymphocytes (PBL). The PBL were then washed, separated from the epithelium and grown for 11 days and shown to be p24 positive by ELISA and were therefore infected (Liu et al., 2003). In 2005, Maher *et al* used tissue explants derived from tonsillectomies in order to study HIV-1 interacting with the tonsil epithelium and resident immune cells. Firstly, they showed HIV-1 infected H9 T lymphocytes bound to the surface of the epithelium and caused p24 accumulation in epithelial explants by immunohistochemistry. They also detected that basal epithelial cells were positive for p24, suggesting transmigration of HIV-1 infected cells or transcytosis of the virus across the epithelium (Maher et al., 2005). Using time lapse confocal microscopy they showed that HIV-1 infected lymphocytes migrated through the epithelium (Maher et al., 2005), thus demonstrating a mechanism by which transmission might occur at the pharyngeal tonsil surface. In 2008, Vacharaksa *et al* used tonsil keratinocytes from donors and OKF6/TERT-2 (TERT-2) cells to *trans*-infect peripheral blood mononuclear cells (PBMCs) after washing. In fact, the TERT-2 cells transferred at least five fold more HIV-1 than donor tissues as demonstrated by p24 ELISA of the PBMCs (Vacharaksa et al., 2008). Giacaman *et al* (2008) followed up soon after and showed that TERT-2 cells pre-stimulated with *Porphyromonas gingivalis* exhibited a greater ability to transfer HIV-1 to TZM-bl than unstimulated virally exposed TERT-2 cells (Giacaman et al., 2008). Thus, despite there being a low apparent *in vivo* transmission rate (Campo et al., 2006), these studies show that *in vitro*, oral and pharyngeal transmission to susceptible cells may occur.

Studies with female genital tract epithelium have also demonstrated its ability to transfer HIV-1 to susceptible cells by capturing or allowing transmigration of infectious virus. In the late nineties, primary PBLs from HIV-1 positive patients were shown to transmit virus across many epithelial lines I407 (intestinal), HT-29 (colonic), Caco-2 (colonic), HEC-1 (endometrial) and infect mononuclear cells on the basolateral side of epithelial barrier (Bomsel, 1997). In 2002, Carreno et al demonstrated with HEC-1 cells that R5 (YU2, JRCSF, BaL) HIV-1 transcytosis was enhanced under pro-inflammatory conditions (Carreno et al., 2002). Furthermore, it was shown that a genital epithelial cell line of ectocervical origin sequestered and transmitted HIV-1 to H9 CD4 positive T cells or PBMC (Wu et al., 2003). Finally, the ability of HIV-1 to retain infectivity after transcytosis across a tight monolayer of primary genital epithelial cells (PGEs) was confirmed by activation of luciferase activity in TZM-bl cells by basally collected medium (Bobardt et al., 2007). However, there are also studies that oppose the view of HIV-1 transmission across the mucosa, since recently HIV-1 was found not to cross the vaginal mucosa within 4 h using a model of a reconstituted human epithelium (Bouschbacher et al., 2008).

Since there is a body of evidence that demonstrated that epithelial cells can transfer HIV-1 to susceptible cells, we aimed to determine whether oral (TR146), oropharyngeal (FaDu) and vaginal (A431) cell lines possessed the ability to transmit HIV-1 to susceptible cells. These cell lines were exposed to HIV-1 YU2 (R5) and LAI (X4) and transfer events were monitored by staining for β -galactosidase activity in the TZM-bl reporter cell line. Qualitatively, transfer was observed at the point of interaction between epithelial cells and surrounding TZM-bl reporter cells, and a similar amount of R5 and X4 viral transfer from all three epithelial cell lines was observed. We conclude that HIV-1 is able to be transmitted from oral and vaginal epithelial cells to susceptible TZM-bl reporter cells.

6.2 Methods

The full methods are detailed in Chapter 2.11.1. Briefly, FaDu, TR146, and A431 cells were plated at low density (50-60% confluence), exposed to 0.2 MOI HIV R5 and X4 overnight, and then washed thoroughly and plated in fresh medium. Freshly grown TZM-bl cells were then overlaid onto the HIV-1 exposed epithelial cells and incubated for a further 48 h. The cells were fixed, stained for β -galactosidase activity with an X-Gal stain and the blue colonies photographed at 200x magnification.

6.3 Results

We wished to verify whether HIV-1 remained infectious while bound to the surface of epithelial cells. We tested this by assessing the ability of epithelial bound virus to trigger β -galactosidase reporter gene expression in engineered reporter cells (TZM-bl). These HeLa derived cells express CXCR4 and were engineered to express CD4, CCR5 (Platt et al., 1998). They were further modified to incorporate reporter cassettes expressing luciferase and β -galactosidase under the control of the HIV-1 LTR where expression of the reporter is triggered by the production of HIV-1 *tat* (Wu X. *et al.*, unpublished data and (Derdeyn et al., 2000)).

All three epithelial lines were able to transfer infectious virus to TZM-bl reporter cells as demonstrated by the blue cells stained for β -galactosidase in Figure 6.1. TZM-bl cells themselves were used as a positive control to show a transmission event from one susceptible cell to another by overlaying unexposed TZM-bl cells onto HIV-1 exposed TZM-bl cells. As expected, a high number of blue colonies were observed. In stark contrast, the negative control comprising virally unexposed epithelial lines overlaid with TZM-bl cells show no blue colonies, as expected. All three epithelial cell lines (FaDu, TR146 and A431) were clearly able to transfer captured YU2-R5 and LAI-X4 HIV-1 to the TZM-bl reporter cell as demonstrated by the blue foci. However, transfer was less common (fewer blue foci) than for the positive TZM-bl control. Notably, there is neither an obvious difference in the efficiency of transmission of R5 or X4 virus nor a difference between the ability of the different cell lines to transmit infectious particles. One striking feature was the ability to observe discrete blue foci at the point of interaction between epithelial colonies and TZM-bl cells (Figure 6.1, black triangles). Further, the negative controls demonstrated that the system is not 'leaky' since virally unexposed epithelial cells were unable to induce blue foci (and thus TZM-bl β -galactosidase activation). Only HIV-1 exposed epithelium triggered the β -galactosidase activity, reporting infection.

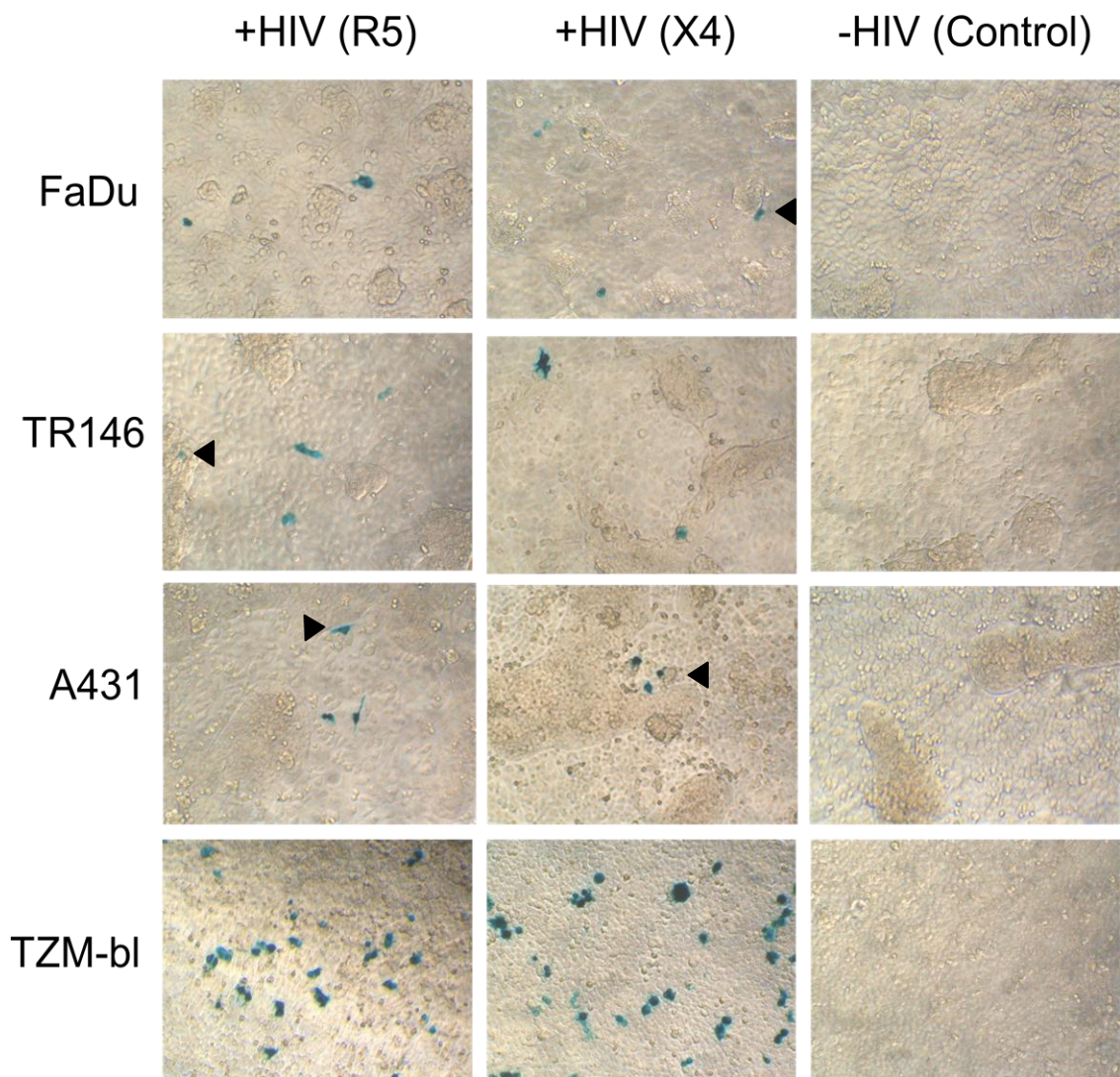


Figure 6-1: Transfer of captured HIV-1 from epithelial cells to permissive cells. TR146, FaDu, A431 and TZM-bl cells were incubated with HIV-1 R5 (YU2) and LAI (X4) virus for 24 h and following extensive washing, TZM-bl indicator cells were added for a further 48 h. Negative controls include TR146, FaDu and A431 cells without the addition of virus, plated with TZM-bl reporter cells. Exposed TZM-bl cells were used as a positive control to indicate transfer between susceptible cells. Black triangles highlight transfer events at the point of contact between epithelial cells and TZM-bl cells. Data are representative of three independent experiments. Light microscopy at 200X magnification.

6.4 Discussion:

In this chapter we determined whether HIV-1 captured by oral and vaginal epithelial cells was transmissible to susceptible TZM-bl reporter cells using β -galactosidase activation as a read out mechanism. In a co-culture assay, HIV-1 was shown to be transmitted from oro-pharyngeal, oral, and vaginal epithelial lines to TZM-bl cells in a manner consistent with previous findings (Giacaman et al., 2008; Liu et al., 2003; Maher et al., 2005; Vacharaksa et al., 2008; Wu et al., 2003). No difference in the

ability of the different lines to transmit virus was observed. Neither did X4 or R5 trophic virus appear to be favoured when passed from the epithelial lines to susceptible cells.

Data obtained in Chapter 4 (section 4.2.1) detecting HIV-1 gp120 on the surface of epithelial lines indicated that pharyngeal (FaDu) cells were more efficient at binding HIV-1 than oral (TR146) and vaginal (A431) cells (~30% versus ~5% respectively). This gave rise to the possibility that more viral transfer might occur from FaDu cells as compared with TR146 and A431 cells, which could be crudely quantified with the TZM-bl transfer assay. However, there appeared to be no obvious difference between the epithelial cell lines in their ability to transfer virus (Figure 6.1), suggesting the efficiency of transfer was equal despite the increase in HIV-1 binding to FaDu cells (Chapter 4).

The most directly comparable study to our work is a publication by Giacaman *et al* (2008) where TERT-2 oral cells (with or without pre-stimulation with *P. gingivalis*) were exposed to HIV-1, washed, trypsinized and then plated onto a TZM-bl cell monolayer. However, unlike our study (Figure 6.1), control wells of TERT-2 without exposure to HIV-1 was either not shown or not performed. Therefore, it was impossible to determine whether β -galactosidase activation (blue colonies) was due to HIV-1 transfer or non-specific activation of the HIV-LTR as a result of cell-cell contact between TERT-2 and TZM-bl reporter cells. In addition, the authors suggested that viral transfer to TZM-bl originates from internalized but still infectious HIV-R5 (Giacaman *et al.*, 2008). This conclusion was based on their previous work using TERT-2 cells where a short, three minute trypsin treatment at room temperature was used to remove virus particles from the epithelial surface before incubation with PBMCs (Vacharaksa *et al.*, 2008). p24 release from the PBMCs was then monitored by ELISA to show the transfer of infectious virus from the TERT-2 to the PBMCs. However, neither removal of HIV-1 from the epithelial surface nor the presence of internalized virus was determined by either flow cytometry or confocal microscopy (e.g. by observing viral p24 colocalization with intracellular compartments). Therefore, it is possible that in the Giacaman *et al* (2008) study, surface associated virus was transferred, as opposed to internalized virus. This is strongly supported by our data in Chapter 4 (Figure 4.4), which showed that trypsin treatment at 37°C for 5 min was insufficient to remove all the bound virus particles from surface of epithelial cells (as detected by gp120 flow cytometry). Therefore, perhaps a more cautious statement indicating that transfer was due to, as they call it themselves, “cell associated trypsin resistant virus” (Giacaman *et al.*, 2008). The aforementioned statement from their paper better reflects their findings and is more consistent with our data. We stress that we have not fully addressed whether HIV-1 transfer is the result of surface bound or internalized virus. However, we strongly suspect, given our data, that transfer is via surface bound virus. Irrespective, since the purpose of this Chapter was to

determine whether HIV-1 can be transferred from epithelial cells to permissive cells *per se*, there was no need to distinguish between these two possibilities.

Other studies have used the p24 accumulation assay (by ELISA) as a measure of infectious HIV-1 transfer to permissive cells but have reached the same conclusions as our study using TZM-bl reporter cells. For example, HIV-1 exposed normal human oral keratinocytes can transfer infectious NL4.3 (X4) and BaL (R5) to PBLs that were lysed for p24 ELISA (Liu et al., 2003). Similarly, genital epithelial cell line Ect1 captured and transmitted many different HIV-1 strains to H9 T cells and IL-2-stimulated PBMC when co-cultured over several days and lysed for p24 ELISA (Wu et al., 2003). The advantage of the p24 ELISA assay is that it is highly quantitative; however, counting plaques in many replicate wells allows more quantitative use of the TZM-bl assay. Irrespective, our data are consistent with these studies and the conclusion is that infectious HIV-1 can be transferred from epithelium to permissive cells.

If epithelial cells can capture and sequester virus even after extensive PBS washing, then *in vivo* salivary flow and vaginal mucous flow may not be sufficient to flush all infectious particles when HIV exposure has occurred. Permissive cells circulating in the sub mucosa may then encounter the bound virus either as it transcytoses the epithelium or when they are recruited by epithelial cells during an inflammatory responses through cytokine and chemokine signals.

6.5 Conclusion

The aim of the chapter was to establish whether oral and vaginal epithelial cells were able to transfer captured infectious HIV-1 to susceptible TZM-bl reporter cells that indicate infection with β -galactosidase activity. We conclude that oro-pharyngeal, oral and vaginal cells transfer infectious HIV-1 to permissive cells, probably from the surface, despite not having an integrated copy of the viral genome (Chapter 5). Thus, epithelium may act as a mediator of systemic infection through transfer of infectious HIV-1 to permissive cells.

Chapter 7 Oral and vaginal epithelial responses to HIV-1

7.1 Introduction

Previously, we found that HIV-1 binds to various oral and vaginal epithelial cell lines (Chapter 4) but does not integrate (Chapter 5) and, therefore, it was concluded that HIV-1 is unable to productively infect epithelial cells. Nevertheless, in Chapter 6, epithelial lines were shown to transfer captured infectious virus to TZM-bl reporter cells. As the literature indicates that epithelial cells can respond to HIV-1 (Ferreira et al., 2011; Nazli et al., 2010), we wished to further characterise this aspect of viral-epithelial interaction. This was achieved by investigating the interactions between HIV-1 envelope protein or whole virus with oral and vaginal epithelium using three separate approaches. First, cytokine production in response to HIV-1 envelope gp120 and multimeric/trimeric gp140 exposure was monitored. Second, activation of intracellular signalling pathways after exposure to whole X4 and R5 virus was assessed by Western blotting. Finally, the global epithelial response in TR146 and primary oral epithelium was determined after exposure to HIV-1 R5 and X4 using Affymetrix human microarrays.

Many studies have treated various cell types with gp120 and observed mixed responses (Del Corno et al., 2005; Kapasi et al., 2002; Martinelli et al., 2007; Shan et al., 2007; Singhal et al., 1999). Most relevant to the current thesis are two studies using kidney epithelium (Kapasi et al., 2002; Singhal et al., 1999). Firstly, gp120 (HIV-1₄₅₁ X4-trophic (Bandres et al., 1998)) was shown to modulate the proliferation of human glomerular epithelial cells by enhancing proliferation at low concentrations (0.1 ng/mL) but decreasing growth and promoting apoptosis at higher concentrations (1-100 ng/mL) (Singhal et al., 1999). The protein kinase C and tyrosine kinase pathways were implicated and expression of c-Jun and c-Fos (members of the AP-1 transcription factor complex that can be activated via one of the three MAPK pathways: p38, JNK and ERK1/2) was increased with high concentration (100 ng/mL) of gp120. Following up, apoptosis caused by gp120 in these cells was shown to be dependent on phosphorylation of p38 and CD4 expression (Kapasi et al., 2002). Thus, in kidney epithelial cells (which express CD4 unlike oral and vaginal epithelial cells) the MAPK pathway has been implicated in responding to HIV-1 gp120.

There are other studies indicating that HIV-1 affects the mucosal barrier. HIV-1 infected individuals have barrier dysfunction in intestinal epithelium which allows bacteria to traverse the mucosa (Epple et al., 2009a; Epple et al., 2009b). To determine whether similar dysfunction can lead to HIV-1 traversing the vaginal epithelium, Nazli *et al.* (2010) monitored the transepithelial resistance (TER), an electrical measurement for the strength of the mucosal epithelial monolayer *in vitro*, and showed a decrease in the strength of the tight junctions in epithelial monolayers and a concordant decrease in tight

junction, RNA and protein expression. Gp120 treatment was sufficient to reduce tight junction protein ZO-1 by fluorescent microscopy and TER measurement in primary endometrial epithelium and an intestinal epithelial line (Nazli et al., 2010). Finally, the authors showed that the cytokines TNF- α , IL-6, IL-10 and IL-1 β were significantly increased in apical medium of ADA (R5 virus) exposed primary endometrial epithelium. These studies strongly suggest that epithelial cells can sense and respond to HIV-1.

There have been a number of publications analysing the global gene response of immune cells to HIV-1 using microarrays. One publication provided an overview of 34 published articles in this area between 2001-2006 (Giri et al., 2006), which included data validated by qPCR or protein expression. They identified many pathways that had been previously reported as being activated or suppressed by HIV-1 or HIV-1 proteins, which included p-38, ERK1/2, Smad3 and NF- κ B, (Acheampong et al., 2005; Cicala et al., 2006; Cicala et al., 2002; Coberley et al., 2004; Guadalupe et al., 2003; Janket et al., 2004; Muthumani et al., 2005; Pulliam et al., 2004; Sankaran et al., 2005; Vazquez et al., 2005; Wen et al., 2005). By extension, one might hypothesize that similar pathways probably play a role in epithelial responses to HIV-1. However, there is a paucity of microarray data for oral and vaginal epithelial cell responses to HIV-1. Only one directly relevant study was performed by Acheampong *et al.* (2005), which used a CLONTECH Atlas human apoptosis cDNA expression array to assess the response of primary oral keratinocytes treated with HIV-1 gp120, Nef and HIV-1 Tat. HIV gp120, Tat and Nef were all found to induce substantial apoptosis at a concentration of 10 ng/ml via the TNF/TNF-R Fas/Fas ligand pathways.

HIV can trigger apoptosis in susceptible cells by four main pathways: extrinsic-induced when a ligand (FasL, TNF or TRAIL) activates a death receptor (TNF receptors) on the cell surface involving caspases, intrinsic mitochondrial induced apoptosis, T-cell mediated cytotoxicity and perforin/granzyme dependent killing of cells (caspase-independent) and autophagy (Février et al, 2011; Cummins et al., 2010). Many components of the virus such as env, Tat, Vpr, Nef are toxic to cells and directly play into the various death pathways even in uninfected cells (Cummins et al., 2010). Therefore, it is possible that HIV could also influence cell death pathways in epithelial lines.

The aim of this chapter was to characterise how oral and vaginal epithelia respond to HIV-1 using TR146 and A431 epithelial cell lines. First, we examined whether HIV-1 env gp120 and gp140 multimer proteins were able to induce cytokine production. Second, we assessed whether epithelial signalling pathways (MAPK (p38, JNK, ERK1/2), NF- κ B and IRF3) were activated by X4 and R5 virus, and looked for cytotoxicity by measuring the damage response as by monitored by the release of lactate dehydrogenase (LDH). Finally, we undertook a preliminary microarray experiment to

characterise the global gene expression profile of TR146 and primary oral epithelial cells treated with R5 and X4 virus. The hypotheses were two fold, (i) that since HIV-1 is able to bind all three epithelial cell lines (Chapter 3), addition of gp120/gp140 alone may be sufficient to elicit cytokine production, and (ii) given that R5 and X4 virus bind predominantly protein and non-protein moieties, respectively (Chapter 4), that there would be differences in the signalling pathway response of oral and vaginal epithelium as well as global gene expression profile of oral epithelium to HIV-1 R5 and X4.

While we found a lack of cytokine (IL-1 α , IL-6, G-CSF, GM-CSF) induction by gp120 or multimeric gp140, there appears to be clear recognition of YU2-R5 and LAI-X4 whole virus by TR146 and A431 cells, since signalling pathways in both epithelial cell types were activated. While TR146 cells appeared less responsive than A431 cells, differences in signalling pathway activation were observed for X4 and R5 virus in both epithelial cell types. This was confirmed in oral epithelial cells at the gene expression level by microarray analysis. Notably, epithelial cell damage was induced by both R5 and X4 virus. Together, the data indicate that R5 and X4 virus are recognized by oral and vaginal epithelial cells but that each virus appears to activate different signalling pathways and gene expression profiles, which may have consequences for R5 and X4 virus infection *in vivo*.

7.2 Methods

7.2.1 Activation of epithelial cytokines by HIV-1 envelope

TR146 and A431 cells were grown to confluence and then serum starved for 2 h prior to stimulation with HIV-1 envelope glycoproteins gp120 BAL-R5 (expression vector by Marvin Reitz), CN54-X4 clade C (Morikawa et al., 1990.) and multimeric/trimeric gp140s UG21-X4 (Jefferies et al., 2004 and 2006) and ZM96-R5 clade C (Rodenburg et al., 2001) at 1 μ g/mL in serum-free medium for 24 h. Supernatants were collected, frozen at -80°C and select cytokines quantified by Luminex multiplex bead assay according to the manufacturer's instructions. The multimeric gp140 UG21 and ZM-96 mammalian glycosylated proteins were produced by mammalian cell transfection rather than in bacteria. UG21 was a kind gift from Dr Simon Jefferies (Imperial College London). Please refer to Chapter 2.9 for full method.

7.2.2 Activation of intracellular signalling pathways in response to HIV-1 in oral and vaginal cells

Epithelial cells were grown to confluence, serum starved overnight, and the next day YU2-R5 and LAI-X4 viruses were added at an MOI 5 for 2 h at 37°C. Conditioned medium controls (culture medium from uninfected cells but grown identically) were added to control wells using the same volume as the medium containing virus particles. Cells were incubated for 2 h, washed with cold PBS, and lysed with cold RIPA buffer

containing Triton X-100, protease and phosphatase inhibitors. The protein extracts were quantified by BCA assay and gels were loaded with equal amounts of protein in each well. Please refer to Chapter 2.5.1 for full method.

7.2.3 Microarray analysis of primary and TR146 epithelial cells treated with HIV-1

TR146 and primary oral epithelial cells were grown to confluence and exposed to HIV-1 LAI-X4 and YU2- R5 virus at an MOI of 100 for 8 h 37°C. Cells were washed thoroughly and RNA isolated for quality control and labelling for microarray analysis by Affymetrix chips. The resulting gene lists were compared with each other to find the gene intersections using Venny (Oliveros, 2007) , a program that creates Venn diagrams from any input lists and the common genes up- or down-regulated in TR146 and primary oral epithelium were analysed using the on-line tool Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al., 2009). Please refer to Chapter 2.10 for full method.

7.3 Results:

7.3.1 Activation of epithelial cytokines by HIV-1 envelope

Since the vagina is a more susceptible milieu for HIV transmission than the oral cavity, we hypothesized that oral and vaginal epithelial cells may produce a different cytokine profile in response to HIV-1 envelope stimulation, which may potentially account for the underlying differences in susceptibility between the two mucosal sites. Therefore, oral and vaginal cell lines were treated with recombinant gp120 (BAL, CN54) and multimeric glycosylated gp140s (UG21, ZM96) to determine whether the HIV-1 envelope protein elicited a cytokine response in epithelial cells after 24 h. However, three independent experiments with A431 cells and two with TR146 cells failed to demonstrate activation of IL-1 α , IL-6, G-CSF or GM-CSF by gp120 or multimeric gp140 at 1 μ g/ml (Fig 7.1 and 7.2). It should be noted that TR146 cells naturally secrete higher amounts of IL-6, G-CSF and GM-CSF than A431 cells.

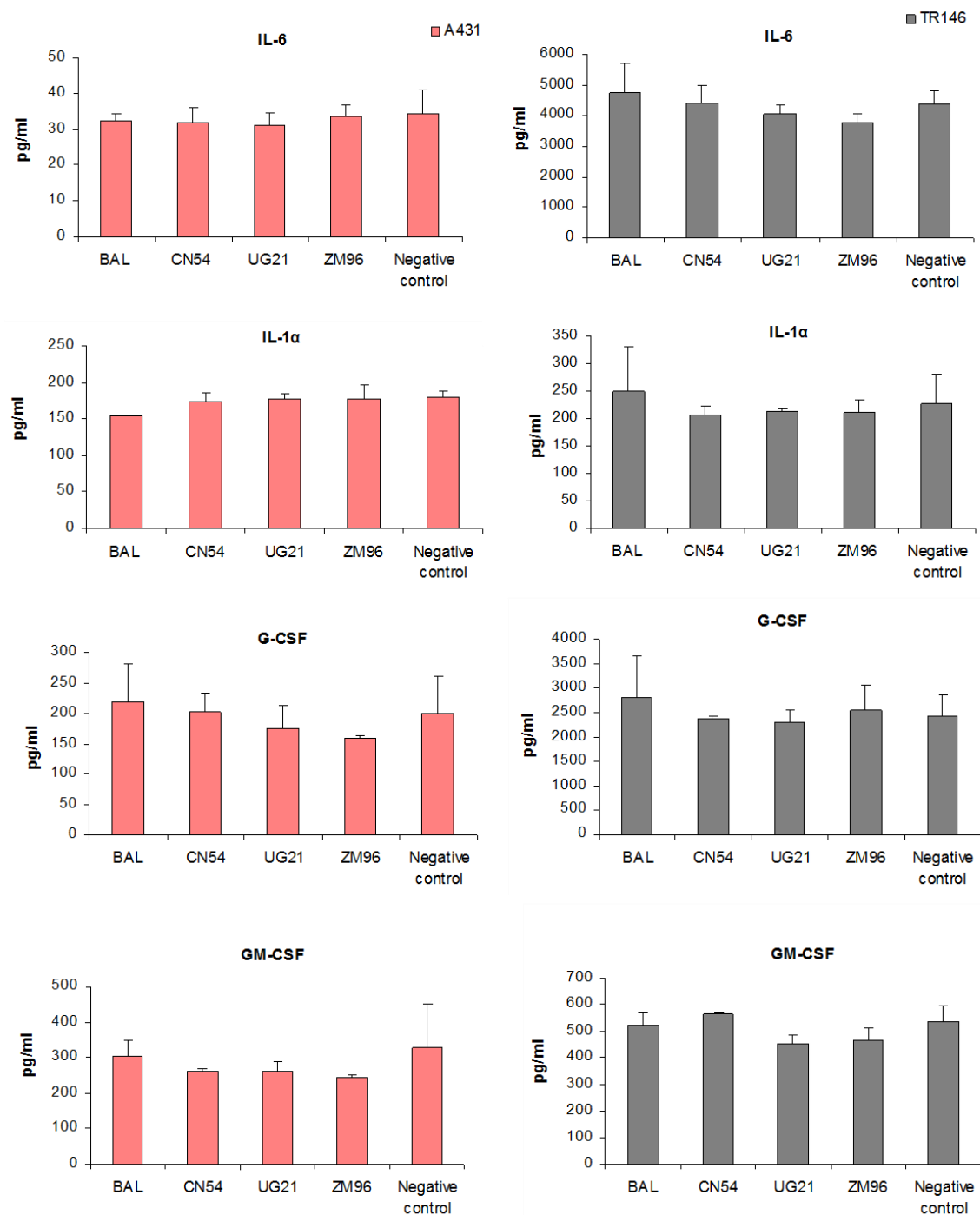


Figure 7-1: IL-6, IL-1α, G-CSF, GM-CSF concentration in culture supernatants from gp120 (BAL, CN54) and gp140 (UG21, ZM96) (1μg) treated epithelial cells as determined by Luminex assay. Two or three technical replicates were averaged and standard deviation shown by error bars. Data are representative of three (A431 in pink) or two (TR146 in grey) independent experiments.

7.3.2 Activation of intracellular signalling pathways in response to HIV-1 in oral and vaginal cells

Our group has previously shown that the oral and vaginal epithelium is responsive to the human pathogenic fungus *Candida albicans* (Moyes et al., 2011; Moyes et al., 2010). The key signalling pathways activated in epithelial cells were found to be MAPK (p38, JNK, ERK1/2) and NF- κ B from this work and our unpublished work on TLR agonists. Activation of epithelial cells by other microbial stimuli, e.g. bacteria and viruses, also demonstrate activation of MAPK and NF- κ B, but also the virally-activated toll-like receptor (TLR)-mediated IRF3 pathway (Carty and Bowie, 2010; Gomez and Prince, 2008; Handfield et al., 2008; Kinane et al., 2008; Mans et al., 2006). Therefore, we decided to monitor MAPK, NF- κ B and the TLR/IRF3 pathways after interactions with HIV-1 at the level of the molecules represented in various shades of purple (Figure 7.2).

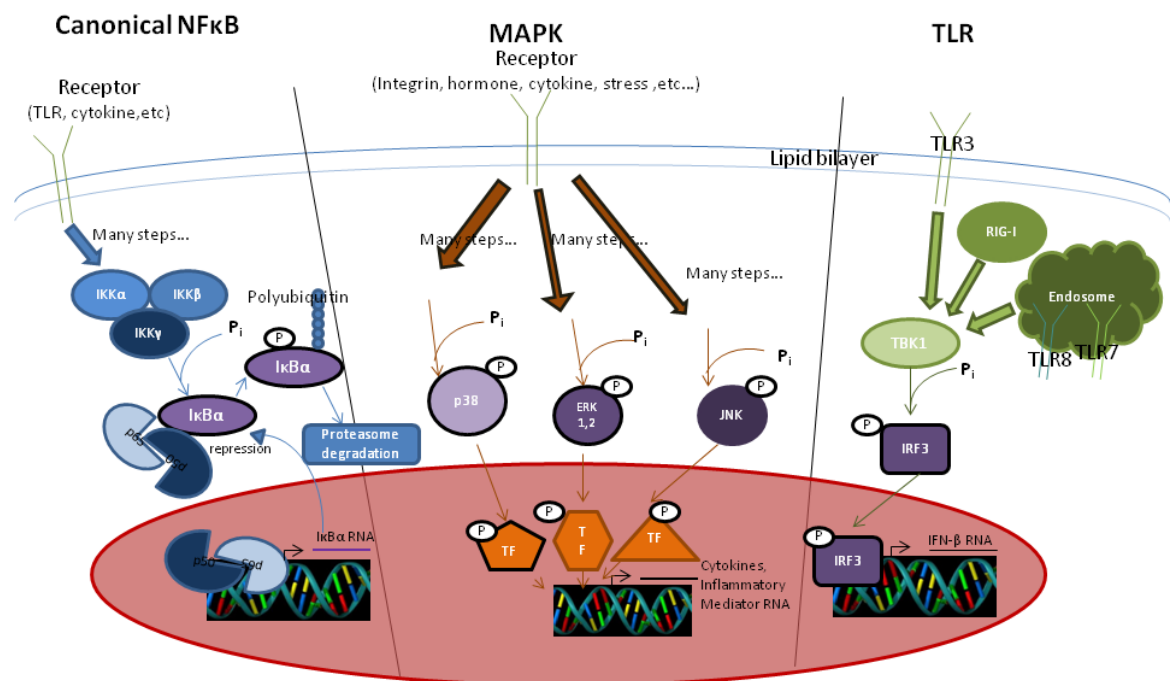


Figure 7-2: Simplified schematic overview of the NF- κ B, MAPK and TLR signalling pathways. Proteins in shades of purple were assessed by Western blotting. See appendix for full pathway diagrams and Literature Review (section 1.8) for more details on the pathways.

TR146 and A431 cells were challenged with R5 (YU2) and X4 (LAI) viruses for 2 h at 37°C and harvested for Western blot analysis. The total protein was isolated and assayed for (i) MAPK pathway activation via phosphorylation of ERK1/2 (p-ERK1/2),

JNK (p-JNK), and p-38 (p-p38) (ii) NF- κ B activation via phosphorylation of the inhibitor I κ B α (p-I κ B α) and (iii) TLR-mediated IRF3 activation via phosphorylation of IRF3 (p-IRF3). The 2 h time point was determined to be optimal in our previous studies investigating the activation of these pathways by western blot in epithelial lines (Moyes et al., 2011 and 2011). The presence or absence of virus in each experimental condition was demonstrated by detecting HIV-1 p24 expression. One strength of our approach was that we compared data from virus treated cells with data from cells treated with conditioned medium from the same source (the virus-producing cell lines without viral production). This provides more confidence that activation is not simply due to a property of the medium containing the virus stock.

Exposure of TR146 cells to HIV-1 R5 and X4 demonstrated differential activation of MAPK signalling as well as NF- κ B signalling (Figure 7.3). For the MAPK pathway, p-ERK1/2 was not activated by X4 virus but was activated by R5 virus, albeit only slightly. However, since the control conditioned medium alone (CX: without X4 virus, and CR: without R5 virus) and the untreated control (U) also activated p-ERK1/2, especially CX, it is difficult to conclude that HIV-1 activates p-ERK1/2. Indeed, one could interpret that X4 virus may in fact inhibit ERK1/2 signalling given the significantly reduced signal compared with conditioned medium alone (CX) and the untreated control. While neither R5, nor X4 virus activated p-JNK, R5 virus did activate p-p38 to a considerable degree over conditioned medium alone (CR). Again, X4 virus may specifically inhibit p-p38 activation as the conditioned medium control (CX) activates p-p38 more strongly than the X4 virus. A similar finding was found for X4 virus activation of NF- κ B in that a fainter band was observed for p-I κ B- α compared with the conditioned medium control (CX). Given the strong signal for p-I κ B- α in the untreated cell control it would appear that p-I κ B- α is not activated by R5 virus. Finally, like p-JNK, a lack of p-IRF-3 signal was observed for all TR146 samples, indicating no activation of the TLR-mediated IRF-3 pathway by HIV-1 virus. Together, the data suggests that in TR146 oral epithelial cells, R5 virus activates the MAPK-p38 pathway whereas X4 virus may potentially suppress p38, ERK1/2 and NF- κ B signalling.

Interestingly, there were both similarities and differences in the A431 vaginal cell response to R5 and X4 virus as compared with TR146 oral cells. Notably, R5 virus activates p-ERK1/2 in comparison with the conditioned medium control (CR) and untreated control, whereas X4 virus may inhibit p-ERK1/2 activation given the significantly reduced signal compared with the conditioned medium control (CX). Also, R5 virus but not X4 virus specifically activates p-JNK and p-IRF3 compared with their conditioned medium controls. Treatment with both R5 and X4 lead to activation of p-p38, but the untreated control signal was relatively high so the data should be interpreted with

caution. As for NF- κ B signalling, there was little observable difference in p-I κ B- α between X4 and R5 virus compared with conditioned medium and untreated controls. Thus, it is difficult to conclude that either X4 or R5 virus activates NF- κ B signalling.

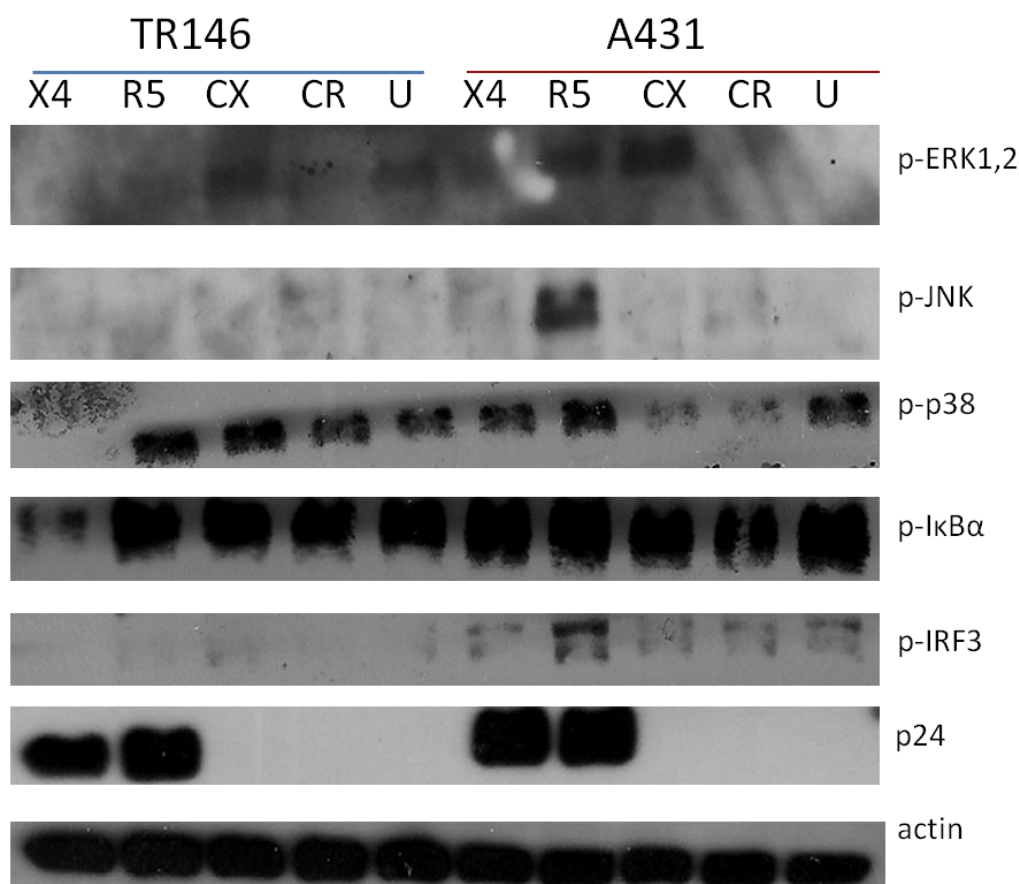


Figure 7-3: Activation of MAPK (ERK1/2, JNK, p38), NF- κ B and IRF3 signalling pathways in TR146 oral and A431 vaginal epithelial cell lines in response to whole virus challenge (MOI of 5). X4: LAI-X4, R5: YU2-R5, CX: control conditioned medium for X4, CR: control conditioned medium for R5, U: untreated cell control. Data are representative of 2-3 independent experiments.

In summary, vaginal and oral epithelial cells respond differently to X4 and R5 whole virus stimulation. Overall, the signalling pathways investigated were activated to a lesser degree in TR146 oral cells than A431 vaginal cells. Notably, R5 virus activates p38 pathway in TR146 cells, whereas X4 virus may inhibit p38 and NF- κ B signalling. Likewise, R5 virus activates ERK1/2, JNK, p38 and IRF3 in A431 vaginal cells, whereas X4 virus appears to only activate p38. Clearly, X4 and R5 viruses activate different signalling pathways in epithelial cells, even when applied at the same MOI.

7.3.2.1 Cell damage

In order to assess cell damage induced by R5 and X4 virus treatment, supernatants were collected and assayed for lactate dehydrogenase (LDH). This

cytoplasmic enzyme is released from cells when the cellular membrane is damaged. We found that both R5 and X4 virus induced LDH release but more release was induced by R5 virus (Figure 7.4), indicating greater damage. Notably, like the untreated cell control, the conditioned medium controls for both virus stocks did not induce LDH release, demonstrating no damage. Since the control conditioned medium was obtained from the same cells used to grow virus stocks, but just without the presence of virus, and are as acidic (by visual inspection of yellow colour), this suggests that damage was induced by the presence of virus.

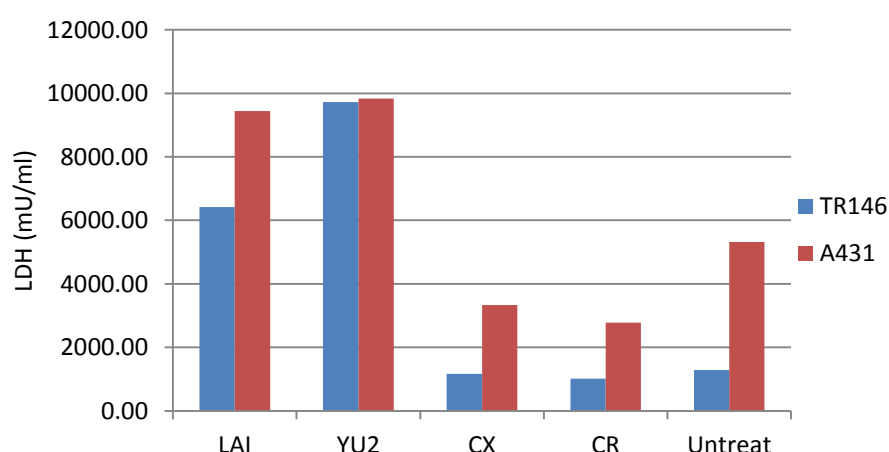


Figure 7-4: Epithelial cell damage induced by R5 and X4 virus as measured by LDH assay in culture supernatants after 24 h. LAI: X4 virus. YU2: R5 virus. LAI C: LAI control conditioned medium. YU2 C: YU2 control conditioned medium. Data are representative of two independent experiments.

7.3.3 Global gene expression responses in oral epithelial cells in response to HIV-1

Given that HIV-1 R5 and X4 bind to oral and vaginal epithelial cells (Chapter 4) and activate signalling pathways (above) we decided to perform a preliminary microarray experiment in primary oral epithelial and TR146 cells to discover how oral epithelium responds on a global scale to challenge with R5 and X4 virus. Although we would also liked to have assessed vaginal epithelial cell responses, especially since A431 cells were more responsive than TR146 cells and provided differences in R5 and X4 virus signalling responses (above), we are unable to obtain primary vaginal epithelial cells as we did not have ethical approval for such studies. Therefore, we proceeded with comparing the gene responses of primary epithelial cells (gingival origin) with TR146 buccal cells, as this would provide valuable information with regard to potential differences in HIV-1 responses between normal and carcinoma epithelial cells. Given the expense of performing microarray work, the limitation of primary epithelial cell numbers, and the

preliminary nature of this aspect of the work, only a singleton array was performed per condition. However, given their singleton nature, caution is advised with regard to interpretation of the data and in drawing specific conclusions. Ideally, for future work multiple samples per condition (3 or more) would need to be assayed. We chose a time point, 8 h, that would allow for intracellular signalling pathways (2-4 h) to have had an effect on gene expression in the epithelial lines as determined by our own unpublished studies (David Moyes, personal communication). Because the effect of a low MOI could be subtle, we chose to use a high MOI in this preliminary, proof of principle study.

In primary oral cells treated with YU2-R5 virus, 1959 genes were up-regulated three-fold or greater versus the untreated cell control, while 1455 genes were down-regulated at least three-fold (Figure 7-5). In the same cells treated with LAI-X4 virus, 936 genes were up-regulated three-fold or greater while 680 were down-regulated at least three-fold. Thus, in primary cells, a greater number of genes changed expression levels in response to R5 versus X4 virus.

In TR146 oral cells treated with YU2-R5, 37 genes are up-regulated three-fold or greater, while 74 genes are down-regulated at least three-fold (Figure 7-5). In the same cells treated with LAI-X4, 20 genes were up-regulated and 15 genes down-regulated at least three-fold. Thus, just like in primary cells, in TR146 cells a greater number of genes changed expression levels in response to R5 versus X4 virus. Notably, the TR146 cell line was less responsive to HIV-1 compared with primary oral epithelial cells. Since only a singleton array was performed for each cell type (HIV-1 R5, X4 or untreated), statistical analysis could not be undertaken; however, the common genes either up- or down-regulated in both TR146 and primary cells are represented in Figure 7-5. Intersecting gene lists were then analysed for gene ontology (biological process, cellular compartment, molecular function and pathway) using DAVID.

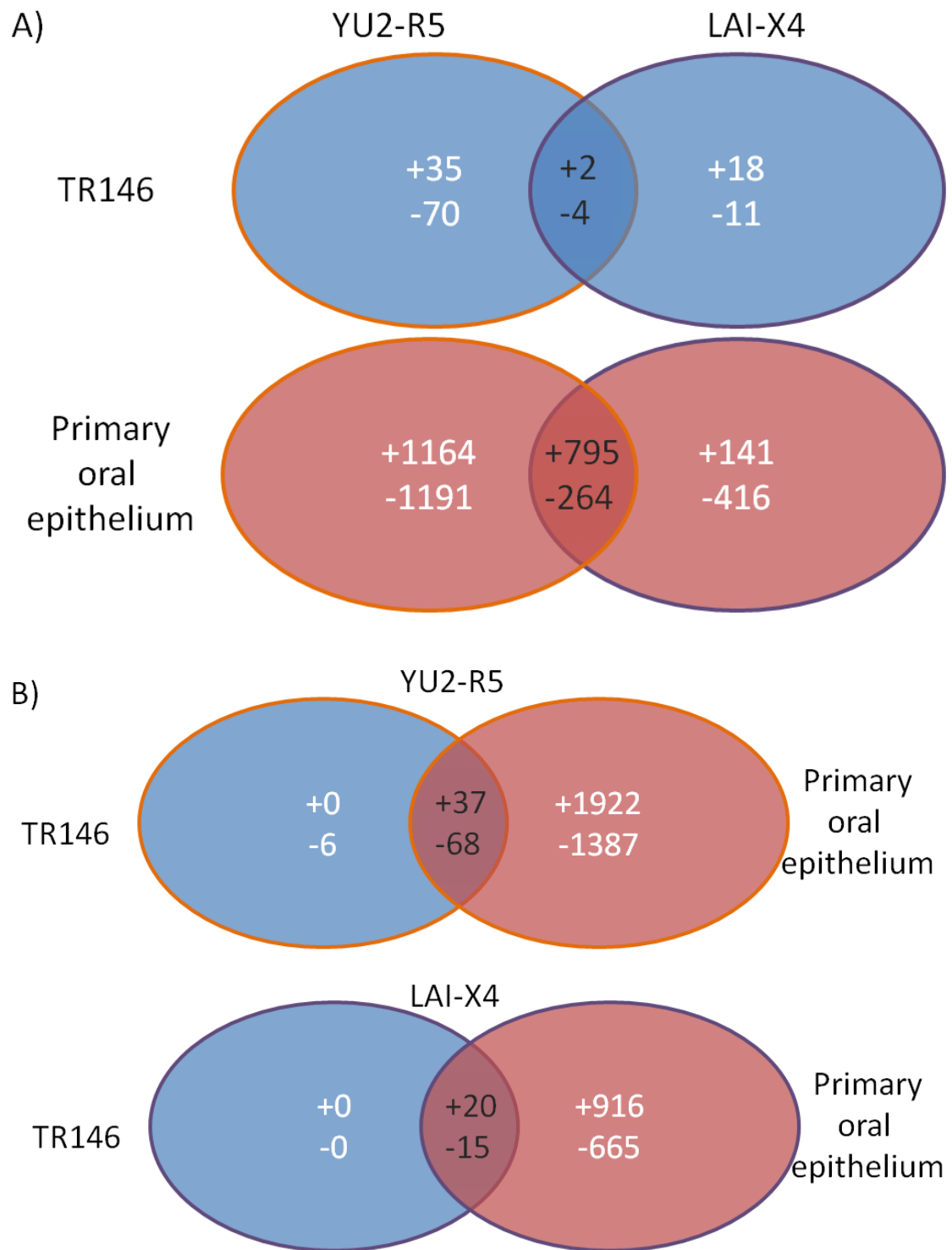


Figure 7-5: Representation of microarray experimental design for microarray experiment. Genes up regulated denoted with +, down regulated with - (3-fold for primary cells and 3-fold for TR146). The number of genes up- and down-regulated in both primary and TR146 cells are shown in the intersection. A) Shows the numbers of genes changed for each cell type and the commonality between treatment with R5 and X4 HIV-1. B) Shows the comparison of the R5 treatment in both cell types versus the X4 treatment in both cell types.

7.3.3.1 Genes up-regulated in primary and TR146 epithelial cells in response to R5 virus

A comparison of the gene lists up and down regulated in TR146 cells and primary oral cells by treatment with HIV YU2-R5 was undertaken. Of the 37 genes increased over three-fold in TR146, all were increased over three-fold in primary oral cells exposed YU2-R5 virus at an MOI of 100. Analysis of this gene list with the DAVID program revealed several pathways appeared activated as compared to a random list of genes in the human genome. The most significant hits ($p < 0.05$) were response to protein stimulus, wound response and positive regulation of apoptosis/programmed cell death. Although no particular cellular compartment was identified, the molecular function with $p < 0.05$ is enzyme binding and the pathway identified is TGF- β signalling.

Table 7-1: Common genes up-regulated in both TR146 and primary oral epithelial cells in response to YU2-R5 after 8h. Entries with most highly significant p-values are displayed.

GO TERM	#	P-Value	Genes
Biological process			
GO:0051789 response to protein stimulus	4	0.001672	heat shock 70kDa protein 8; inhibitor of DNA binding 3, dominant negative helix-loop-helix protein; cysteine-rich, angiogenic inducer 61; inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
GO:0009611 response to wounding	5	0.028476	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein; vanin 1; plasminogen activator, urokinase receptor; serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1; transferrin receptor (p90, CD71)
GO:0042981 regulation of apoptosis	6	0.030273	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein; aryl-hydrocarbon receptor repressor; programmed cell death 6; vanin 1; RAS p21 protein activator (GTPase activating protein) 1; eukaryotic translation initiation factor 5A-eukaryotic translation initiation factor 5A-like 1; inhibin, beta A
GO:0043067 regulation of programmed cell death	6	0.03142	List same as above
GO:0010941 regulation of cell death	6	0.031858	List same as above
Cellular compartment			
None specified			
Molecular function			
GO:0019899 enzyme binding	6	0.006435	junction plakoglobin; Rho-related BTB domain containing 3; plasminogen activator, urokinase receptor; RAS p21 protein activator (GTPase activating protein) 1; serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1; glutaredoxin 3
KEGG pathway			
hsa04350: TGF- β signalling pathway	3	0.026297	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein; inhibitor of DNA binding 1, dominant negative helix-loop-helix protein; inhibin, beta A

#: number of genes implicated in list for association with particular function or pathway.

p-value: (or EASE score) is a modified Fisher Exact Probability P-value. The smaller it is the more significant it is.

H_0 = the gene list is not enriched for given pathways versus a random list from the genome.

7.3.3.2 Genes down-regulated in primary and TR146 epithelial cells in response to R5 virus

When genes down-regulated at 3 fold level by R5 treatment are considered in both the primary oral and TR146 cells, a list of 68 common genes are identified. Negative regulation of apoptosis and programmed cell death emerge in the “top ten” biological processes (Table 7.2). Also significant ($p < 0.05$) but not included in the “top ten” are phosphorylation, positive regulation of notch signalling, regulation of cell adhesion/migration, integrin mediated signalling, intracellular signalling cascade (Table 7.2). Cellular compartments that are implicated are the cytosol, endosome, transcription factor complex and the microtubule cytoskeleton. Finally, molecular functions identified include integrin binding, microtubule binding, serine threonine kinases and the Wnt signalling pathway (Table 7.2).

7.3.3.3 Genes up-regulated in primary and TR146 epithelial cells in response to X4 virus

Only twenty common genes were up-regulated in TR146 and primary oral epithelial cells when incubated with X4-LAI virus. Given such a few number of genes were identified, it was difficult to establish gene network relationships with certainty. However, the top significance biological processes were distinct from those elicited by R5 virus and included: response to bacteria, negative regulation of MAPK, regulation of phosphorylation, and transcription (Table 7.3). However, similarities were also observed between R5 and X4 virus, including cell cycle regulation. Interestingly, just above the level for significance were: cytokine mediated signalling pathway ($p = 0.0505$) and response to cytokine stimulus ($p = 0.056$). Although no genes were significant for molecular function, the MAPK pathway was implicated (Table 7.3).

Table 7-2: Common genes down-regulated in both TR146 and primary oral epithelial cells in response to YU2-R5 after 8 h. "Top ten entries" with most highly significant p-values are displayed.

GO TERM	%	PValue	Genes
Biological process			
GO:0043066 negative regulation of apoptosis	9	1.4E-04	superoxide dismutase 2, mitochondrial; mitogen-activated protein kinase kinase kinase 7; protein kinase C, iota; similar to Rho-associated, coiled-coil containing protein kinase 1; Rho-associated, coiled-coil containing protein kinase 1; tumor protein p63; prion protein; interleukin 1, alpha; superoxide dismutase 2, mitochondrial; transcription factor 7-like 2 (T-cell specific, HMG-box); S-phase kinase-associated protein 2 (p45)
GO:0043069 negative regulation of programmed cell death	9	1.55E-04	List identical to above
GO:0060548 negative regulation of cell death	9	1.58E-04	List identical to above
GO:0006916 anti-apoptosis	7	2.67E-04	superoxide dismutase 2, mitochondrial; protein kinase C, iota; tumor protein p63; prion protein; interleukin 1, alpha; superoxide dismutase 2, mitochondrial; transcription factor 7-like 2 (T-cell specific, HMG-box); S-phase kinase-associated protein 2 (p45)
GO:0048598 embryonic morphogenesis	7	0.002134	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1); matrix metalloproteinase 13 (collagenase 3); mitogen-activated protein kinase kinase 7; tumor protein p63; transcription factor 7-like 2 (T-cell specific, HMG-box); neurofibromin 2 (merlin); pre-B-cell leukemia homeobox 2.
GO:0042981 regulation of apoptosis	11	0.002297	mitogen-activated protein kinase kinase kinase 7; superoxide dismutase 2, mitochondrial; similar to Rho-associated, coiled-coil containing protein kinase 1; Rho-associated, coiled-coil containing protein kinase 1; tumor protein p63, jun oncogene; interleukin 1, alpha; prion protein; transcription factor 7-like 2 (T-cell specific, HMG-box); protein kinase C, iota; mitogen-activated protein kinase 9; superoxide dismutase 2, mitochondrial; S-phase kinase-associated protein 2 (p45)
GO:0043067 regulation of programmed cell death	11	0.00247	List identical to above list
GO:0010941 regulation of cell death	11	0.002537	List identical to above list
GO:0030036 actin cytoskeleton organization	6	0.002994	Dystonin; protein kinase C, iota; similar to Rho-associated, coiled-coil containing protein kinase 1; Rho-associated, coiled-coil containing protein kinase 1; PDZ and LIM domain 7 (enigma); v-crk sarcoma virus CT10 oncogene homolog (avian); neurofibromin 2 (merlin)
GO:0051726 regulation of cell cycle	7	0.003105	hect domain and RLD 5; jun oncogene; interleukin 1, alpha; cyclin E2; transformation/transcription domain-associated protein; G-2 and S-phase expressed 1; S-phase kinase-associated protein 2 (p45)

GO TERM	%	PValue	Genes
Cellular compartment			
GO:0005829 cytosol	13	0.00839	mitogen-activated protein kinase kinase kinase 7; coiled-coil containing protein kinase 1, jun oncogene, cyclin E2; transcription factor 7-like 2 (T-cell specific, HMG-box); ATPase, H ⁺ transporting, lysosomal 70kDa, V1 subunit A; ribosomal protein S11 pseudogene 5; ribosomal protein S11; protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1); C-terminal binding protein 1; protein kinase C, iota; 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble); 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; v-crkl sarcoma virus CT10 oncogene homolog (avian)
GO:0005768 endosome	6	0.01038	low density lipoprotein receptor; protein kinase C, iota; CAP-GLY domain containing linker protein 1; phosphatidylinositol 4-kinase, catalytic, beta; v-crkl sarcoma virus CT10 oncogene homolog (avian); neurofibromin 2 (merlin)
GO:0005667 transcription factor complex	5	0.0118	C-terminal binding protein 1; jun oncogene; transformation/transcription domain-associated protein; transcription factor 7-like 2 (T-cell specific, HMG-box); pre-B-cell leukemia homeobox 2
GO:0015630 microtubule cytoskeleton	7	0.02754	Dystonin; similar to Rho-associated, coiled-coil containing protein kinase 1; Rho-associated, coiled-coil containing protein kinase 1; CAP-GLY domain containing linker protein 1; tubulin, delta 1; myristoylated alanine-rich protein kinase C substrate; G-2 and S-phase expressed 1; transducin (beta)-like 1 X-linked receptor 1
Molecular function			
GO:0005178 integrin binding	3	0.03036	Dystonin, intercellular adhesion molecule 1, ADAM metalloproteinase domain 10
GO:0019992 diacylglycerol binding	3	0.03938	protein kinase C, iota; similar to Rho-associated, coiled-coil containing protein kinase 1; Rho-associated, coiled-coil containing protein kinase; protein kinase C, eta
GO:0008017 microtubule binding	3	0.04367	Dystonin; CAP-GLY domain containing linker protein 1; prion protein
GO:0004674 protein serine/threonine kinase activity	6	0.04808	mitogen-activated protein kinase kinase kinase 7; protein kinase C, iota; similar to Rho-associated, coiled-coil containing protein kinase 1; Rho-associated, coiled-coil containing protein kinase 1; serine/threonine-protein kinase QSK; mitogen-activated protein kinase 9; protein kinase C,
KEGG pathways			
hsa04310: Wnt signalling pathway	7	4.19E-04	C-terminal binding protein 1; mitogen-activated protein kinase kinase kinase 7; similar to Rho-associated, coiled-coil containing protein kinase 1; Rho-associated, coiled-coil containing protein kinase 1; jun oncogene; mitogen-activated protein kinase 9; transcription factor 7-like 2 (T-cell specific, HMG-box); transducin (beta)-like 1 X-linked receptor 1
hsa05200: Pathways in cancer	8	0.00515	C-terminal binding protein 1; jun oncogene; mitogen-activated protein kinase 9; cyclin E2; transcription factor 7-like 2 (T-cell specific, HMG-box); v-crkl sarcoma virus CT10 oncogene homolog (avian); S-phase kinase-associated protein 2 (p45); fumarate hydratase
hsa05120: Epithelial cell signalling in Helicobacter pylori infection	4	0.01018	jun oncogene; mitogen-activated protein kinase 9; ATPase, H ⁺ transporting, lysosomal 70kDa, V1 subunit A; ADAM metalloproteinase domain 10
hsa04510: Focal	5	0.04350	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4

adhesion			receptor); similar to Rho-associated, coiled-coil containing protein kinase 1; Rho-associated, coiled-coil containing protein kinase 1, jun oncogene; mitogen-activated protein kinase 9; v-crkl sarcoma virus CT10 oncogene homolog (avian)
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#: number of genes implicated in list for association with particular function or pathway.

p-value: (EASE score) if H_0 is that the gene list is not enriched for a given pathways versus a random list from the genome.

Table 7-3: Common genes up-regulated in both TR146 and primary oral epithelial cells in response to LAI-X4 after 8 h. Entries with most highly significant p-values are displayed.

GO TERM	#	P-Value	Genes
Biological process			
GO:0009617 Response to bacterium	3	0.0085	interleukin-1 receptor-associated kinase 3; jun oncogene; chemokine (C-C motif) ligand 20
GO:0010605 negative regulation of macromolecule metabolic process	4	0.0143	NACC family member 2, BEN and BTB (POZ) domain containing; interleukin-1 receptor-associated kinase 3; jun oncogene; hypermethylated in cancer 2
GO:0051726 regulation of cell cycle	3	0.0236	jun oncogene; interleukin 1, alpha; cyclin G2
GO:0043407 negative regulation of MAP kinase activity	2	0.0263	dual specificity phosphatase 6; interleukin-1 receptor-associated kinase 3; dual specificity phosphatase 6
GO:0045449 regulation of transcription	6	0.0279	NACC family member 2, BEN and BTB (POZ) domain containing; interleukin-1 receptor-associated kinase 3; jun oncogene; zinc finger and BTB domain containing 6; hypermethylated in cancer 2; zinc finger protein 14
GO:0042325 regulation of phosphorylation	3	0.0444	dual specificity phosphatase 6; interleukin-1 receptor-associated kinase 3; jun oncogene
GO:0019220 regulation of phosphate metabolic process	3	0.0477	List identical to above list
Cellular Compartment			
GO:0005654 nucleoplasm	3	0.0413	NACC family member 2, BEN and BTB (POZ) domain containing; dual specificity phosphatase 6; jun oncogene
Pathways			
hsa04010:MAPK signaling pathway	3	0.0247	dual specificity phosphatase 6; jun oncogene; interleukin 1, alpha

#: number of genes implicated in list for association with particular function or pathway.

p-value: (EASE score) if H_0 is that the gene list is not enriched for a given pathways versus a random list from the genome.

7.3.3.4 Genes down-regulated in primary and TR146 epithelial cells in response to X4 virus

Only 16 common genes were down-regulated in TR146 and primary oral epithelial cells when incubated with LAI-X4 virus. Given so few genes were identified, it was difficult to establish the network relationships with certainty. Only data for the cellular compartment involved are listed in Table 7.4.

Table 7-4: Common genes down-regulated in both TR146 and primary oral epithelial cells in response to LAI-x4 after 8 h. "Top ten entries" with most highly significant p-values are displayed.

GO TERM	#	P-Value	Genes
GO:0043232 intracellular non-	9	0.001377	ribosomal protein L17 pseudogene 22; ribosomal protein L17 pseudogene 36; ribosomal protein L17 pseudogene

membrane-bounded organelle			20; similar to ribosomal protein L17; ribosomal protein L17 pseudogene 33; ribosomal protein L17 pseudogene 34; ribosomal protein L17 pseudogene 9; ribosomal protein L17; ribosomal protein L17 pseudogene 18; ribosomal protein L17 pseudogene 7; ribosomal protein L17 pseudogene 39; junction plakoglobin; heterogeneous nuclear ribonucleoprotein A1-like 3; similar to heterogeneous nuclear ribonucleoprotein A1; heterogeneous nuclear ribonucleoprotein A1 pseudogene 2; heterogeneous nuclear ribonucleoprotein A1; heterogeneous nuclear ribonucleoprotein A1 pseudogene; small proline-rich protein 2B; keratin 23 (histone deacetylase inducible); histone cluster 2, H2aa3; histone cluster 2, H2aa4; cyclin A1; inhibitor of DNA binding 2, dominant negative helix-loop-helix protein; activating transcription factor 3
GO:0043228 non-membrane-bounded organelle	9	0.001377	As above
GO:0005829 cytosol	5	0.038752	ribosomal protein L17 pseudogene 22; ribosomal protein L17 pseudogene 36; ribosomal protein L17 pseudogene 20; similar to ribosomal protein L17; ribosomal protein L17 pseudogene 33; ribosomal protein L17 pseudogene 34; ribosomal protein L17 pseudogene 9; ribosomal protein L17; ribosomal protein L17 pseudogene 18; ribosomal protein L17 pseudogene 7; ribosomal protein L17 pseudogene 39; junction plakoglobin; heterogeneous nuclear ribonucleoprotein A1-like 3; similar to heterogeneous nuclear ribonucleoprotein A1; inhibitor of DNA binding 2, dominant negative helix-loop-helix protein; aspartyl-tRNA synthetase

#: number of genes implicated in list for association with particular function or pathway.

p-value: (EASE score) if H_0 is that the gene list is not enriched for a given pathways versus a random list from the genome.

7.4 Discussion:

The aim of this Chapter was to characterise how oral and vaginal epithelia respond to R5 and X4 virus, which may help us understand the underlying difference in susceptibility of these respective mucosal sites to HIV-1 infection. We hypothesised that differences may exist in the epithelial responses to R5 and X4 virus, since R5 and X4 virus may to bind different surface moieties as suggested by varying degrees of trypsin sensitivity of HIV binding epithelium (Chapter 4.3.2).

First, we determined whether HIV-1 envelope proteins gp120 (BAL, CN54) and gp140 (Ug21, ZM96) were able to induce cytokines in TR146 oral or A431 vaginal epithelial cells but found that none of the proteins tested were able to induce IL-6, IL-1 α , G-CSF or GM-CSF in these cell lines. This was, in part, unexpected since Nazli *et al.* (2010) showed cytokine induction (TNF- α , IL-6, IL-10, IL-1 β) for HIV-1 ADA (R5) treated primary endometrial cells and determined that transepithelial resistance and ZO-1 tight junction protein expression was gp120-dependant. We expected that IL-6 would be produced and act as our positive control. We did not test for TNF- α or IL-10 since neither TR146 or A431 cells secreted these cytokines when exposed to multiple stimulators,

including TLR1-9 agonists (unpublished) and the fungal pathogen *C. albicans* (Moyes et al., 2010). However, we do show that HIV-1 gp120 as well as (mammalian produced) glycosylated gp140s do not induce four key cytokines known to be secreted by these oral and vaginal cell lines (Moyes et al., 2010) and which are involved in initiating immune responses (Weindl et al., 2007). The differences in cytokine induction between our work and that of Nazli et al. (2010) most likely stem from the different origin of the epithelium used (endometrial versus oral/vaginal), the primary (Nazli) versus carcinoma (our work) status of the cells, and the fact we utilized purified envelope protein as opposed to whole virus. It would have been ideal to test whole virus exposed supernatants for cytokine induction but this was not possible for technical reasons, as the Luminex machine was located outside of the Category 3 facility. To remove HIV-1 exposed samples from the Category 3 facility requires either heat inactivation (60°C for 10 min - 1 h) or fixation (1% glutaraldehyde or 1-2% formaldehyde) of the virus. However, pilot studies showed that these treatment regimes denatured the cytokines in the culture supernatants thereby diminishing our ability to detect them by luminex with any accuracy (data not shown). Alternative approaches including inactivation of HIV-1 by UV treatment were also unsuccessful (data not shown).

Second, we investigated the ability of R5 and X4 virus to activate signalling pathways in TR146 and A431 epithelial cells. The reasoning behind this stemmed from our findings in Chapter 4, where we found that R5 and X4 virus appear to bind different surface moieties on epithelial cells. Thus, we hypothesised that R5 and X4 virus may activate different signalling pathways in oral and vaginal epithelial cells. Also, since primary endometrial cells produce cytokines in response to ADA (R5) virus (Nazli et al., 2010), then intracellular signalling cascades are clearly activated in epithelial cells in response to HIV-1. An assortment of signalling pathways were targeted based on unpublished results from our group where agonists of TLR3 and TLR7/8 activated MAPK, NF- κ B, IRF3 (David Moyes, personal communication). These pathways were also known to be implicated in HIV-1 responses in immune cells and therefore were targeted in these preliminary studies. Western blotting was used to visualize their active phosphorylated forms. We found that (i) In TR146 cells, R5 virus activates the p38 pathway whereas X4 virus may inhibit p38, ERK1/2 and NF- κ B signalling, (ii) In A431 cells, R5 virus activates ERK1/2, JNK, p38 and IRF3 whereas X4 virus appears to only activate p-38, and (iii) that A431 vaginal cells were more responsive to HIV-1 than TR146 oral cells. Although the implication of NF- κ B and MAPK pathways in HIV-1 responses is consistent with previous microarray studies (Acheampong et al., 2005; Cicala et al., 2006; Cicala et al., 2002; Coberley et al., 2004; Guadalupe et al., 2003; Janket et al., 2004; Muthumani et al., 2005; Pulliam et al., 2004; Sankaran et al., 2005; Vazquez et al., 2005; Wen et al., 2005), there is a lack of data regarding epithelial

signalling responses to HIV-1. Since epithelial cell recognition of microbial pathogens are known to differ from that of immune cells (i.e. dendritic cells, T-cells, macrophages), it is difficult to interpret our findings in the context of the current literature. Only two studies were found in relation to our work, both using kidney epithelium. The first study demonstrated that gp120 was able to activate the protein kinase C and tyrosine kinase pathways and implicated the transcription factors c-Jun and c-Fos (via the MAPK pathway) in their activation (Singhal et al., 1999). Our data using whole virus demonstrated activation of all three MAPK pathways (ERK1/2, JNK, p38), predominantly by R5 virus in A431 vaginal cells. However, whether MAPK activation resulted in c-Jun and c-Fos activation is unknown, although both these transcription factors are known to be activated in TR146 and A431 cells in response to fungal pathogens (Moyes et al., 2011; Moyes et al., 2010). The second study showed that apoptosis caused by gp120 in kidney epithelium was dependent on p38 activation (Kapasi et al., 2002). Although we did not investigate apoptosis, interestingly we did find that in A431 cells both R5 and X4 virus activated p38, whereas in TR146 cells only R5 virus activated p38 with X4 virus potentially inhibiting p38 signalling. Thus, the data may suggest that R5 potentially promotes apoptosis in both oral and vaginal epithelial cells, whereas X4 promotes apoptosis only in oral epithelial cells and may inhibit apoptosis in vaginal epithelial cells. However, significant additional work would be required to determine if this hypothesis has merit. It would have been worthwhile considering apoptosis assays such as Invitrogen ApoDETECT AnnexinV-FITC flow alongside detection of membrane integrity or ApoTarget™ Caspase Colorimetric Protease Assay Sampler Kit (caspase-2, caspase-3, caspase-6, caspase-8, caspase-9). Alternatively, R&D systems offer a TUNEL assay to detect fragmented DNA, the hallmark of apoptosis, with incorporation of labelled TdT. Future work should also address the outcome of these signalling pathways looking at TNF, interferon, interferon stimulated genes and other relevant cytokines.

We also assessed for the ability of R5 and X4 virus to induce epithelial damage by monitoring LDH release into the culture medium and found that both R5 and X4 virus supernatant induced LDH release from TR146 and A431 cells, suggestive of cell damage. This data is, in part, consistent with cell death/apoptosis induction as a result of gp120 treatment of kidney epithelium and oral epithelium (Acheampong et al., 2005; Kapasi et al., 2002; Singhal et al., 1999). However, it should be stressed that since the viral stocks used in this study remained in the medium when these supernatants were analysed, it is possible that some/all of the LDH detected originated from the virus-producing cells and not epithelial lines. In addition, we found that R5 induces more damage than X4 virus in both TR146 and A431 cells. This was very intriguing and may be potentially explained by the fact that R5 virus activated more signalling pathways in A431 cells than X4 virus and in the case of TR146 cells was less inhibitory than X4 virus.

Finally, we performed a preliminary exploratory microarray experiment in order to explore the global changes in gene expression that occurred in oral epithelial cells by assessing the transcriptional profile of TR146 and primary oral epithelial cells in response to R5 and X4 virus. Since only singleton arrays were used, this analysis can only give an indication of the oral epithelial response to HIV-1 rather than a complete analysis, which would require triplicates for each experimental condition followed by ANOVA analysis with multi-test correction statistical methods. However, although the data do not comply with MIAME guidelines for publication (Brazma et al., 2001), they do provide some interesting preliminary findings. Notably, that in both TR146 and primary oral cells, the number of genes altered by YU2-R5 virus was greater than that of LAI-X4 virus. This is consistent with a microarray study in PBMCs showing distinct differences in responses to X4 and R5 gp120 (Cicala et al., 2006). Our findings may be explained by the fact that R5 virus predominantly binds protein moieties on the surface of the tested epithelial cell lines whilst X4 virus predominantly binds non-protein moieties (Chapter 4.3.2). It also potentially fits with the idea that R5-trophic viruses might be more inflammatory since they are generally responsible for founding initial HIV-1 infections *in vivo* (Xiao et al., 2000).

We also found that more genes are altered in expression in primary gingival cells as compared with TR146, which may seem surprising since TR146 cells are considered to be in a less regulated state, being a carcinoma cell line. However, TR146 cells have become lab-adapted and therefore may be less responsive than primary cells in this context. This highlights the fact that carcinoma cell lines are not a perfect substitute for primary oral cells in infection research (Moharamzadeh et al., 2012; Yadev et al., 2011). However, due to availability and ease of manipulation, carcinoma cell lines can provide information that can then be verified in primary cells that are more scarce and challenging to culture. It would be ideal to repeat western and microarray experiments with primary cells from relevant sites originating from several donors with several virus isolates.

Finally, we found that while R5 and X4 virus activate different functional gene groups in TR146 cells, the majority of genes activated by both virus tropisms in primary oral epithelium are common (although there are twice as many genes activated by R5). This finding may be explained by the different origins of the cells (TR146 buccal versus primary gingival) as well as the carcinoma status of TR146 cells (above). Irrespective, it would appear that distinct signalling pathways may be activated in TR146 cells in response to R5 and X4 viruses, whereas common pathways are activated in primary oral epithelium. This is supported by the western blot signalling data showing that, unlike R5 virus, X4 virus may specifically inhibit p38 and NF- κ B signalling, which may also

contribute to the decreased number of genes found altered by X4 virus as compared with R5 virus. Furthermore, there is consistency between the array and signalling data sets. For example, inhibition of p38 signalling by X4 virus in TR146 cells by Western correlated well with “negative regulation of MAPK” in the arrays with X4. We are further comforted by the fact that the pathways identified (MAPK, NF- κ B) are similar to those found to be regulated in other HIV related microarray studies in different cell types (Acheampong et al., 2005; Cicala et al., 2006; Cicala et al., 2002; Coberley et al., 2004; Giri et al., 2006; Guadalupe et al., 2003; Janket et al., 2004; Muthumani et al., 2005; Pulliam et al., 2004; Sankaran et al., 2005; Vazquez et al., 2005; Wen et al., 2005). In addition, a validation of the finding that apoptosis pathways may be affected by YU2-R5 could be achieved by using the R&D systems human apoptosis antibody array that would allow the quantification of 35 apoptosis genes in one sample. This would allow the identification of the type of apoptosis involved in HIV exposure of the epithelium.

Recombinant envelope proteins did not elicit cytokines, while whole virus did, as shown by the microarray data. This can be explained by the fact that a great excess of whole virus was used in the microarray study. Also, whole virus contains many more protein components toxic to cells (Tat, Nef, Env) as compared to recombinant envelope which is purified.

In summary, our data suggests that oral and vaginal epithelium act as immune sensors as well as a barriers to infection, since the signalling and gene pathways activated are similar to those implicated in the response of immune cells to HIV-1 infection. However, with regard to the array work, a fully controlled study with both oral and vaginal epithelial cells and different R5 and X4 viruses is required to fully ascertain the global gene expression profile of mucosal epithelium in response to HIV-1.

Chapter 8 Interactions between HIV-1 and *Candida*

8.1 Introduction

In the previous chapters the interaction between oral and vaginal epithelial cells and HIV-1 has been explored. However, aside from (host) epithelial cells, the other main cellular constituents of mucosal surfaces are resident microbiota. The epithelium is populated by diverse commensal microbiota that can turn pathogenic and cause inflammation under suitably predisposing conditions or when homeostasis is disturbed. The last few years has seen an increase in research attention given to co-pathogens and their effect on HIV-1 infection. The presence of co-pathogens can lead to changes in gene expression that appears to promote HIV-1 infection by attracting immune cells and increasing inflammatory cytokines. For example, *Neisseria gonorrhoeae* was shown to induce the expression of human β -defensin-5 and 6, leading to an increase in HIV-1 infectivity since defensins attract dendritic cells that can subsequently be exposed to HIV-1 present in the vaginal epithelium (Klotman et al., 2008). In other work, supernatants collected from endometrial epithelial cells infected with HSV-1/2, *N. gonorrhoeae*, and TLR ligands FimH (TLR4), flagellin (TLR5), and Poly (I:C) (TLR3) induced HIV-LTR reporter gene expression (Ferreira et al., 2011). Thus, inflammation induced by co-pathogens in the vaginal milieu may have an effect on HIV-1 infection by altering host gene expression and inflammatory mediators of epithelial cells.

The idea that oral microbiota can promote HIV-1 disease has also gained popularity. Infection with the periodontal pathogen *Porphyromonas gingivalis* correlates with increased CCR5 expression on oral and tonsil epithelial cells, promoting receptor-mediated recognition and binding of HIV-1 and transfer to susceptible immune cells (Giacaman et al., 2008). Also, bacterial extracts from oral pathogens *Fusobacterium nucleatum* and *P. gingivalis* were shown to induce the release of inflammatory signals that could lead to HIV-1 reactivation (Gonzalez et al., 2010). In addition, *F. nucleatum* and *P. gingivalis* were shown to activate NF- κ B and increase cytokine expression (Milward et al., 2007), therefore potentially having an additive effect in promoting HIV-1 infection in the oral cavity (Chapter 7.3.2). Thus, the human microbiota may stimulate inflammation that, in turn, may promote activation of HIV-1 gene expression and the exposure of susceptible immune cells to the virus in the mucosa.

Oral microbiota may also bind to HIV-1 directly (Gruber et al., 2003) and protect the virus from being cleared by the flow of saliva and mucosal secretions, potentially facilitating transfer. One such microbe is *Candida albicans*, an opportunistic fungus that causes oral candidiasis in ~50% of untreated HIV-infected subjects and ~90% of AIDS patients (Phelan et al., 1987). In two studies, the HIV-1 gp41/gp160 envelope protein

was shown to directly bind *C. albicans*, increase *C. albicans* adhesion to HIV-1 infected cells, decrease phagocytosis of the yeast form, and increase secreted aspartyl protease release (a fungal virulence factor) (Wurzner et al., 1997) (Gruber et al., 1998). The same authors also claimed that HIV-1 Tat alone induced hyphal growth in *C. albicans* and increased phagocytosis (Gruber et al., 2001). However, another group showed that a small peptide portion of Tat entered and accumulated in *C. albicans* yeast cells and inhibits hyphal formation by inhibiting the cell cycle in G1 phase (Jung et al., 2006). In addition, apart from *C. albicans*, HIV-1 gp41 has been shown to bind other *Candida* species including *C. dubliniensis* and *C. tropicalis*. gp41 is able to modulate *C. albicans* adhesion to HIV-1 infected T cells as well as uninfected epithelial and endothelial cells (Gruber et al., 2003). Most recently, a complement receptor 3 (CR3) analogue in *C. albicans* (Hgt1) was identified that interacts with HIV-1 (Lesiak-Markowicz et al., 2011), although the evidence provided for a direct interaction is tenuous. Thus, our current understanding of *Candida*-HIV-1 interactions is elementary and somewhat confusing.

Work with HTLV and biofilm formation indicates that HTLV forms a carbohydrate (glycosylated) “cocoon” around itself in order to bind host cells and evade the immune system, thereby enhancing infectious capacity and improving transmission within the host (Pais-Correia et al., 2010; Thoulouze and Alcover, 2011). Like HTLV, HIV-1 is a simian derived retrovirus with two single stranded RNAs as its genome. Although work with HIV-1 in this area has not been undertaken, it is possible that given the highly glycosylated nature of gp120, HIV-1 may bind to biofilms laid down by the host microbiota including the carbohydrate/polysaccharide moieties of *Candida* species. In nature, the majority of the molecular mass of HIV-1 gp120 is made up of N-linked glycans (Allan et al., 1985; Checkley et al., 2011). *Candida* also has abundant glycosylation sites for attachment of N-linked and O-linked glycans, and many mutants defective in N- and O- glycosylation have been developed to assess cell-cell interactions (Bates et al., 2006; Bates et al., 2005; Munro et al., 2005). Thus, glycosylation moieties may be involved in HIV-1 and *C. albicans* interactions.

The aims of this chapter were to determine (i) whether exposure of oral and vaginal epithelial cells to *C. albicans* alters the gene expression of HIV-1 associated receptors, thereby potentially promoting viral binding to epithelial cells, (ii) whether HIV-1 directly binds *Candida* species and can be transferred to susceptible reporter cells, and (iii) whether the fungal moieties that bind HIV-1 and potentially mediate viral transfer. We investigated these by real-time PCR, p24 Western blotting, transfer of infectious virus to TZM-bl cells, and the utilisation of *C. albicans* glycosylation mutants and purified cell wall polysaccharide moieties. We determined that (a) there were minimal alterations in HIV-1 receptor gene expression when epithelial cells were exposed to *C. albicans*, (b) HIV-1

directly binds *Candida* species and can be transferred to susceptible cells, and (c) fungal chitin and β -glucan may be responsible for HIV-1 binding but only chitin appears to permit viral transfer to susceptible cells.

8.2 Methods

8.2.1 HIV related gene expression in epithelial lines following yeast stimulation

For HIV related gene expression in epithelial lines following stimulation with SC5314 *C. albicans* (see section 2.4.2.1), A431 and TR146 cell monolayer were stimulated for 24h with 2×10^4 SC5314 yeast cells (2 yeast per 100 cells or MOI 0.02) and then RNA harvested and qRT-PCR performed as detailed in Chapter 2.4.2 (also in Table 2.1 and 2.2).

8.2.2 Yeast binding to HIV-1 and transfer of infectious HIV-1

For yeast binding p24 western and yeast transfer to TZMbl cells, refer to 2.1.8.2 and 2.1.15, respectively. Briefly, *C. albicans* and other *Candida* and yeast species cells were grown overnight at 30°C in YPD with shaking and 1×10^5 cells were exposed to approximately one infectious viral particle per cell (MOI confirmed as indicated) and incubated with shaking at 30°C for the indicated amount of time. Cells were centrifuged and washed thoroughly, and then either diluted with Laemmli's buffer for boiling and Western blot analysis or incubated in DMEM 10 with amphotericin B (a fungicide) and plated onto TZM-bl cells in 96 well plates. Cells were fed with additional medium containing amphotericin B before being fixed and stained for β -galactosidase (blue foci).

8.3 Results

8.3.1 HIV-1 receptor gene expression in oral and vaginal epithelial cells in response to *C. albicans*.

The resting expression levels of the HIV-1 receptor-associated genes are shown in Figure 8.1 and closely mirror those found in Chapter 3 (Fig. 3.1). Briefly, TR146 cells only express high levels of *SDC-1*, whereas A431 cells express moderate levels of *SDC-1* and *ICAM-1*. The HIV-1 canonical receptors *CD4*, *CCR5*, *CXCR4* (and *DC-SIGN*) were very lowly expressed or undetectable. *SDC-4* was also lowly expressed in both epithelial cell types. After exposure to *C. albicans* SC5314 for 24 h, the expression of all genes did not alter dramatically. Although some genes appear to demonstrate a decrease in expression in one or both cell types, the data is skewed since it derives from expression levels that are already very low and thus cannot be deemed relevant. In conclusion, no major changes in HIV-1 gene receptor levels occur in response to *C. albicans* SC5314 at 0.02 MOI

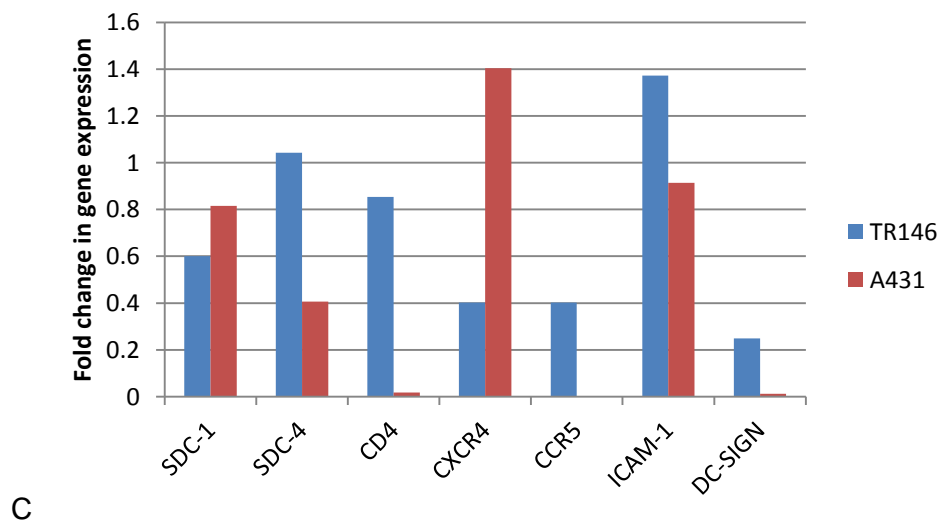
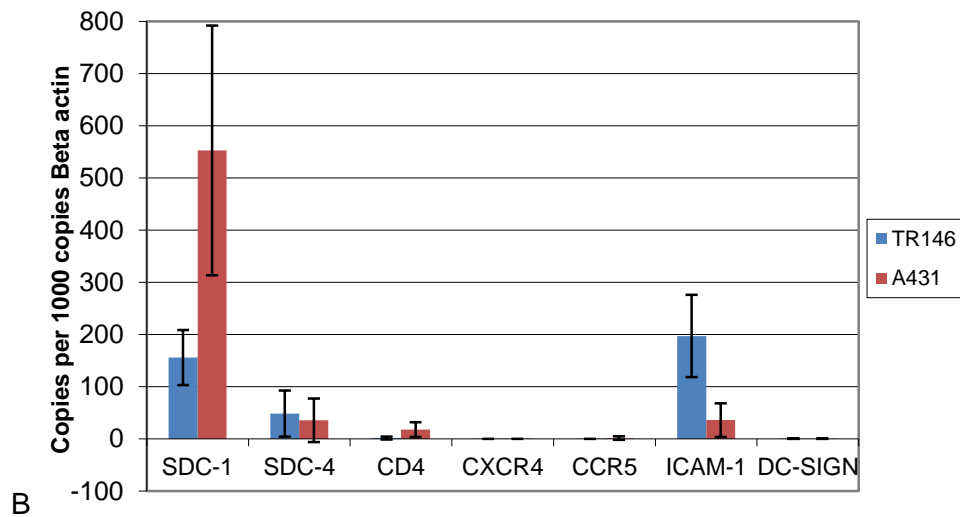
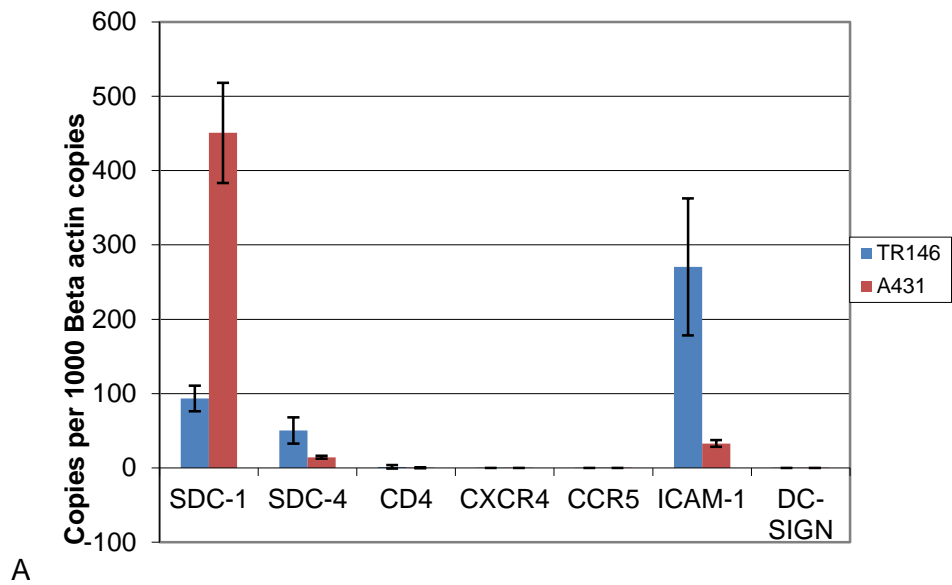


Figure 8-1: Gene expression of HIV-1 associated receptors in oral TR146 and vaginal A431 epithelial cells. HIV-1 associated receptor gene expression by qPCR at rest (A), after C.

albicans SC5314 24 h incubation (yeast MOI of 0.02) (B), and fold change after 24 h treatment incubation with *C. albicans* (C). Graphs are representative of three independent experiments, each performed in duplicate and quantified using three technical replicates.

8.3.2 Binding of HIV-1 to *C. albicans*

Although binding of HIV-1 gp41 by different species of *Candida* has been previously reported (Gruber et al., 2003), the binding of whole R5 and X4 virus to *Candida* has not previously been investigated to our knowledge. Thus, we incubated different *Candida* species (two strains of *C. albicans* and *C. dubliniensis*, *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. guilliermondii* and *Saccharomyces cerevisiae*, see Methods 2.11) for 24 h with YU2-R5 and NL4.3-X4 virus and assessed for the presence of HIV-1 p24 by Western blot (Figure 8.2). It should be noted that for this experiment the MOIs of the two viruses were not the same (MOI of 8 for X4 and MOI of 0.5 for R5 or 8×10^5 X4 virus added versus 5×10^4 R5 virus added). Irrespective, when the viruses are considered individually, X4 virus appears to bind the two *C. albicans* strains most strongly, followed by *C. tropicalis*, then *C. glabrata* and *S. cerevisiae* equally well, and finally the remaining *Candida* species less strongly. Therefore, X4 virus binds to all the fungal species tested. Although the MOI of the R5 virus was lower, HIV-1 p24 could still be detected for all fungal species with strongest binding to *C. albicans* SC5314. Interestingly, binding to another *C. albicans* strain (529L) was as weak as with the other fungal species. Furthermore, although *C. dubliniensis* has been exposed to higher titre of X4 virus, the p24 band for R5 appears to be stronger, although this may be an anomaly.

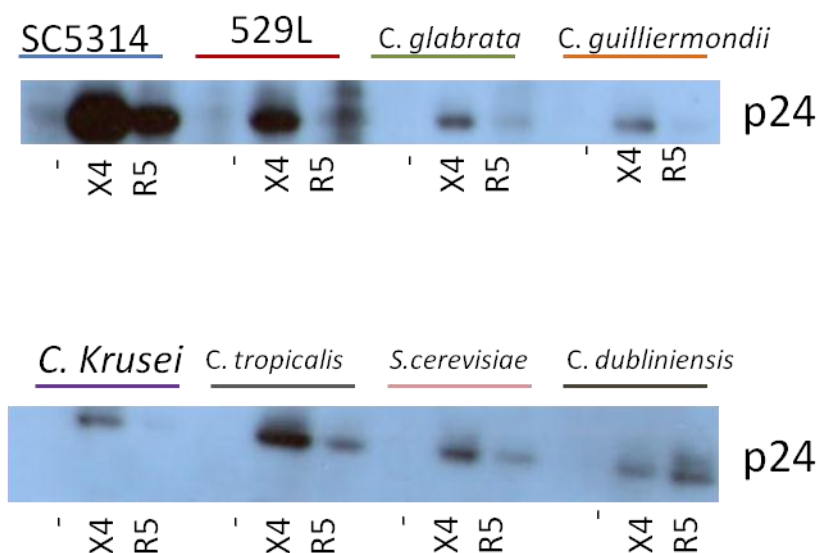


Figure 8-2: Detection of HIV-1 p24 on different fungal species. Different fungal species were incubated with R5 and X4 virus for 24 h at 30°C and p24 detected by Western blot. Data are representative of 2 independent experiments.

Given that R5 is the tropism that normally initiates primary systemic HIV-1 infections, all future work was performed using R5 virus. However, for the repeat binding experiment we used a different R5 virus (JRCSF-R5) and decreased the length of incubation to 2 h. We did this for two reasons; first, to determine whether viral binding would occur in a shorter time frame than 24 h and, second, that the poor detection of YU2-R5 may be due to removal of this virus after 24 h by unknown fungal mechanisms. We found that JRCSF-R5 virus bound strongly to all fungal species by 2 h, with *C. guilliermondii* having the poorest signal for p24 by Western blot (Figure 8.3).

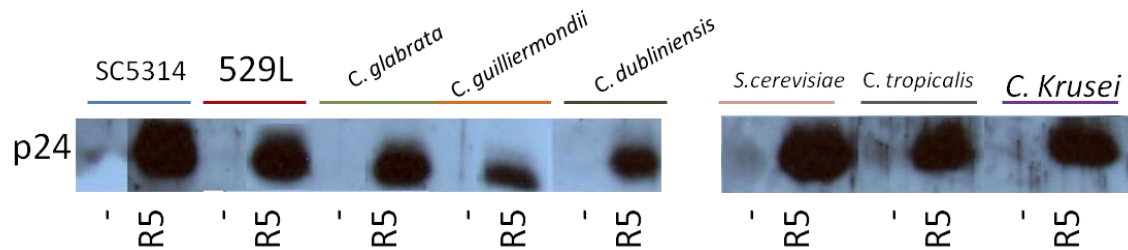


Figure 8-3 Detection of HIV-1 p24 on different fungal species. Different fungal species were incubated with JRCSF-R5 virus for 2 h at 37°C and p24 detected by Western blot. Data representative of 2 experiments.

Given that we showed HIV-1 can be transferred from epithelial cells to permissive cells (Chapter 6), we hypothesized that HIV-1 may also be transferred from *Candida* (e.g. from resident microbiota *in vivo*) to permissive cells. To test this, an assay was developed with *C. albicans* to determine whether this fungus could bind HIV-1 R5 and X4 virus and then release infectious virus to permissive TZM-bl reporter cells. For this assay to be successful, either Fungizone or Amphotericin B (antifungal agents), was required to be added to the culture medium in order to prevent *C. albicans* from over-growing and killing the TZM-bl cells before blue foci (representing infection) could be evaluated. Figure 8.4 shows the presence of blue colonies 48 h after *C. albicans* (SC5314) with bound HIV-1 was placed onto the TZM-bl cells, indicating that the HIV-LTR driven reporter was activated, resulting in gene β -galactosidase expression. Therefore, *C. albicans* can bind and transfer HIV-1 R5 and X4 virus to permissive host cells.

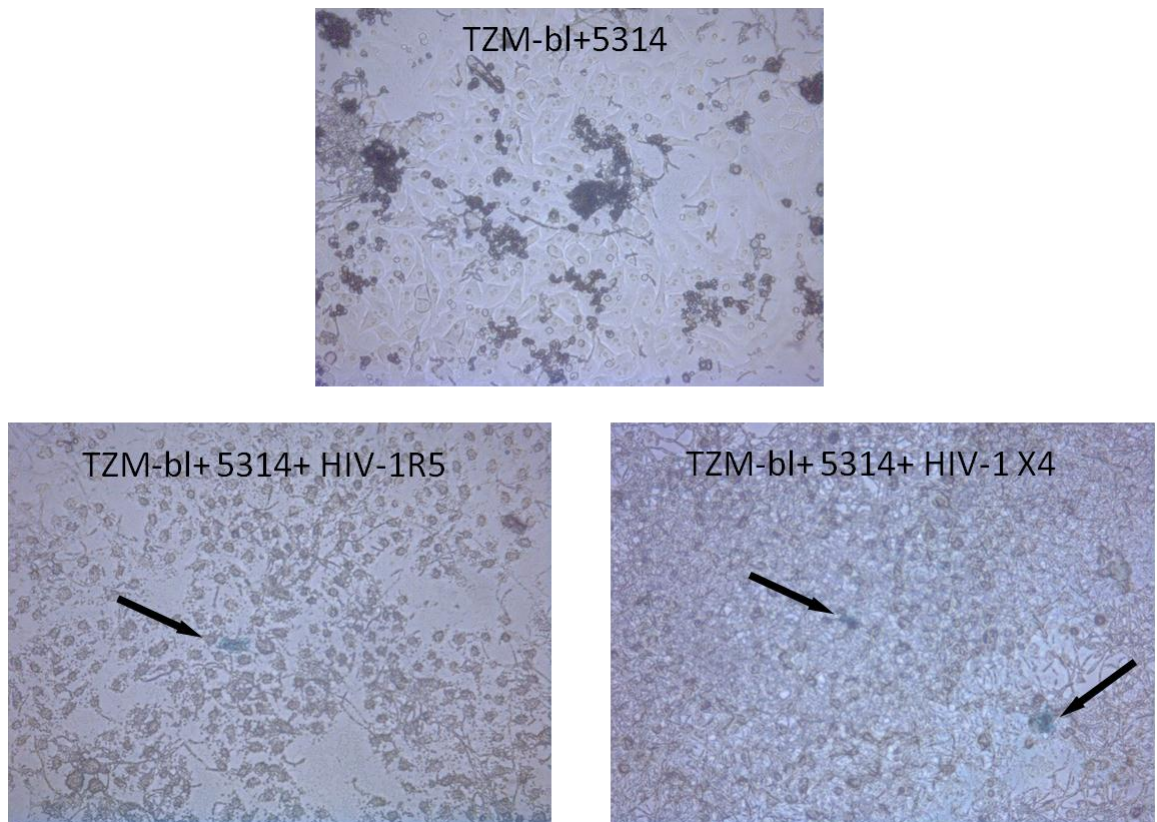


Figure 8-4: Transfer of HIV-1 from *C. albicans* to permissive TzM-bl cells. *C. albicans* was incubated with HIV-1 YU2-R5 and NL4.3-X4 overnight, washed thoroughly, and plated onto TzM-bl cells in the presence of Fungizone. Blue colonies are shown by arrows and represent activation of TzM-bl cells by visualising for β -galactosidase activity. Data are representative of 5 independent experiments.

Next, we wished to determine whether HIV-1 could bind to both yeast and hyphal forms of *C. albicans* and whether the virus could be transferred from both morphologies to susceptible cells. To test this, we incubated *C. albicans* mutants locked in the yeast ($\Delta eed1$) (Zakikhany et al., 2007), pseudohyphal ($\Delta tup1$) (Braun and Johnson, 1997) and hyphal ($\Delta nrg1$) (Murad et al., 2001) (see 2.11) growth phases with YU2-R5 virus for 24 h, followed by incubation with TzM-bl cells for 48 h. Figure 8.5 shows that all *C. albicans* mutants as well as the wild type SC5314 were able to bind R5 virus (p24 detection) and transfer virus to TzM-bl reporter cells. Therefore, viral transfer was independent of *C. albicans* morphology. However, it was noted that the two hyphal producing strains (wild type SC5314 and $\Delta nrg1$) transferred fewer viral particles than the non-hyphal forming strains ($\Delta eed1$ and $\Delta tup1$).

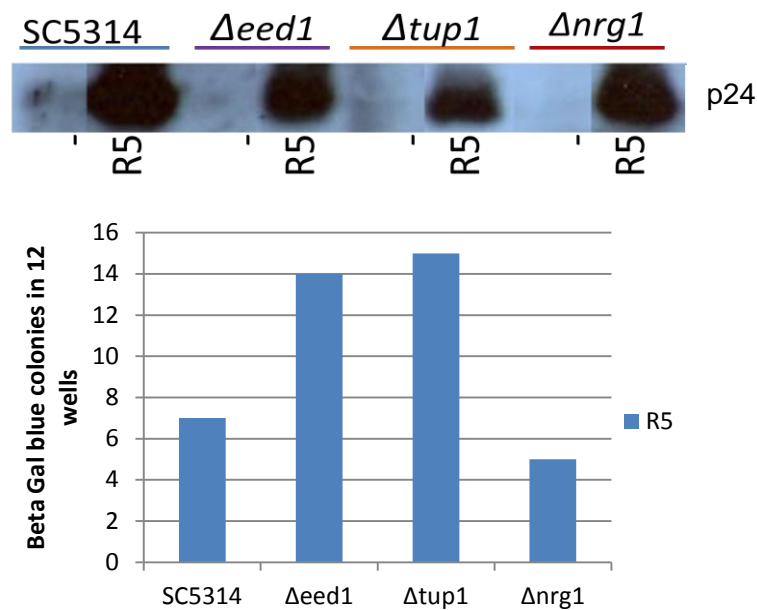


Figure 8-5: *C. albicans* yeast and hyphae bind and transmit R5 virus to TZM-bl reporter cells. Yeast locked $\Delta eed1$, pseudohyphal locked $\Delta tup1$ and hyphal locked $\Delta nrg1$. MOI =1. Top panel shows p24 western: R5 shows YU2-R5 exposed yeast and – represents untreated yeast control. Bottom panel shows yeast transfer of infectious HIV-1 to TZM-bl reporter cells. Data are representative of two experiments.

Since all the *Candida* strains and yeast/hyphal locked mutants were able to bind HIV-1, this indicated that the virus probably binds cell surface structures common to all fungi. The cell wall of fungi including *C. albicans* (yeast and hyphae) is composed of three main components: chitin, β -glucan and proteins that are highly N- and O-glycosylated (Chaffin, 2008). Since the outer most layer is formed of highly glycosylated proteins, we first tested whether *C. albicans* mutants defective in N- and O- protein glycosylation lost their ability to bind HIV-1 and transfer virus. We utilised three key *C. albicans* mutants known to be defective in either N- glycosylation ($\Delta och1$) (Bates et al., 2006), O- glycosylation ($\Delta mnt1/2$) (Munro et al., 2005), or both N- and O-glycosylation ($\Delta pmr1$) (Bates et al., 2005) (see section 2.11). Figure 8.6 shows a schematic representation of the sugars/glycans affected in these genetic mutants (Murciano et al., 2011). However, we found that all the glycosylation mutants were able to bind (Figure 8.7) and transfer (Figure 8.8) YU2-R5 virus as well as the parent *C. albicans* strain (CAI4).

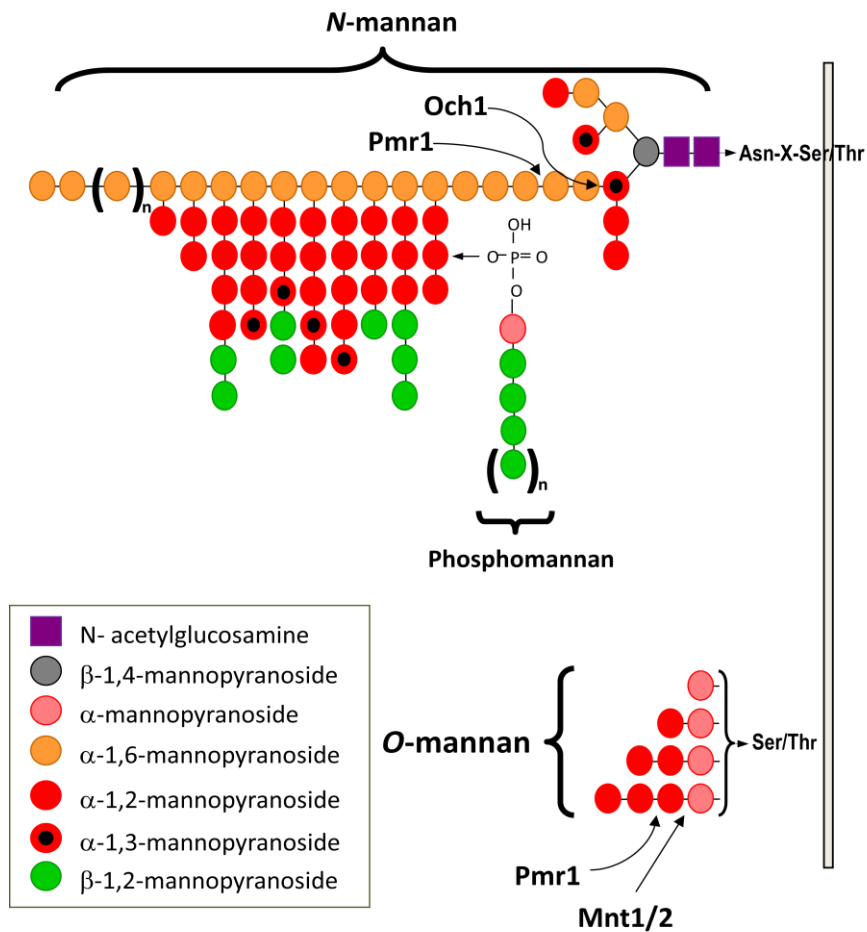


Figure 8-6: Schematic representation of N- and O- glycosylation gene activity in *C. albicans*. Gene activity shown for Pmr1, Och1 and Mnt1/2. Adapted from (Murciano et al., 2011).

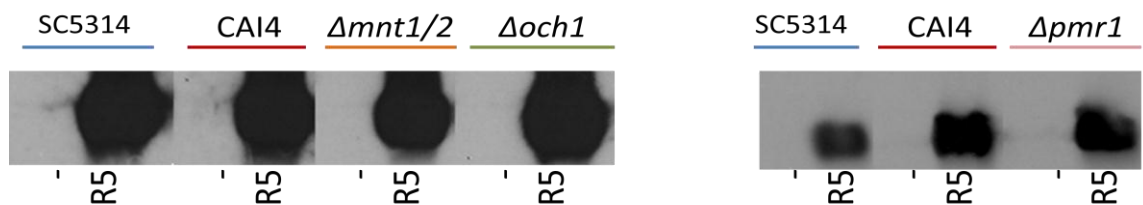


Figure 8-7: Detection of HIV-1 p24 on different *C. albicans* glycosylation mutants. *C. albicans* glycosylation mutants were incubated with YU2-R5 virus for 24 h at 30°C and p24 detected by Western blot. Data are representative of 2 independent experiments.

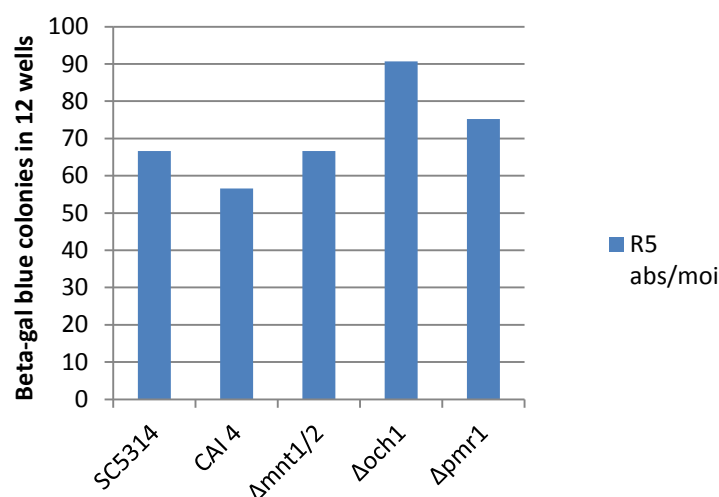


Figure 8-8: *C. albicans* glycosylation mutants can transfer YU2-R5 virus to TZM-bl reporter cells. HIV-YU2 MOI 1.29. Data are representative of 3 independent experiments.

Our data indicated that HIV-1 is unlikely to be binding to the glycosylation residues of surface proteins in *C. albicans*. Thus, we next investigated whether HIV-1 was able to bind to the other two main constituents of the *C. albicans* cell wall: chitin and β -glucan. Another reason for testing this was because of our data demonstrating that the two hyphal producing strains (wild type and $\Delta nrg1$) appeared to transfer fewer viral particles than the non-hyphal forming strains ($\Delta tup1$ and $\Delta eed1$) (Fig 8.5). Although *C. albicans* hyphae possess more chitin than yeast cells the chitin is not as exposed as in yeast cells (in yeast cells chitin is exposed in bud scars during separation of the mother and daughter cell) (Munro et al., 1998). Thus, we hypothesised that if chitin was involved in HIV-1 binding and transfer, then the lack of exposed chitin on the surface of hyphal cells may explain why the two hyphal producing strains transferred fewer viral particles than the non-hyphal forming strains (Fig 8.5).

Thus, we obtained purified chitin (crab) and β -glucan (David Williams, University of Tennessee) and investigated their ability to bind and transfer YU2-R5 virus. This was undertaken together with the N- and O-glycosylation mutants for comparison. We also wanted to include purified N- and O-mannan in these studies rather than the glycosylation mutants; however, the purified mannan was soluble, unlike the chitin and β -glucan which were provided as insoluble particulate material. Establishing an assay to test HIV-1 binding to soluble mannan was not possible within the time frame of this thesis. However, performing these experiments with chitin and β -glucan was rapid since the insoluble particulates could easily be centrifuged and washed after exposure to HIV-1, unlike the mannan preparations.

Figure 8.9 shows that YU2-R5 virus binds strongly to both chitin and β -glucan, at levels similar to *C. albicans* wild type (SC5314) and parent (CAI4) strains. Since HIV-1 binding was approximately equivalent to that of three glycosylation mutants (which do not possess N- or O-mannan), we tentatively concluded that HIV-1 probably binds to the chitin and β -glucan components of the *C. albicans* cell wall and is unlikely to bind the glycosylation moieties of surface proteins. However, confirmation of this using purified mannan is required.

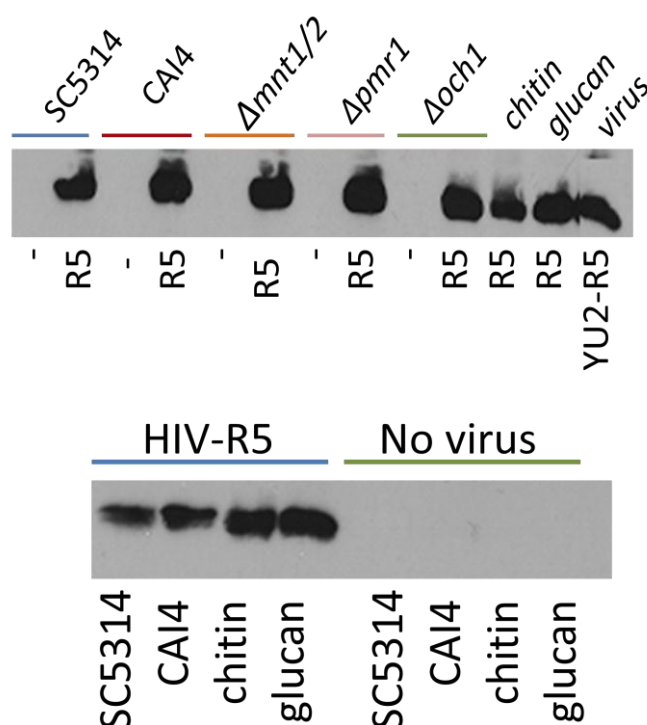


Figure 8-9: Detection of HIV-1 p24 to *C. albicans* chitin, β -glucan and different glycosylation mutants. YU2-R5 virus was incubated with *C. albicans* chitin, β -glucan and different glycosylation mutants for 24 h at 30°C and p24 detected by Western blot. Data are representative of 2-4 independent experiments.

We then investigated whether both chitin and β -glucan could transfer infectious R5 virus to TZM-bl reporter cells. Very interestingly, Figure 8.10 shows the number of blue colonies per well (MOI=145) and that HIV-1 can be efficiently transferred from chitin but not from β -glucan when compared with the wild type *C. albicans* SC5314. Therefore, β -glucan transfers HIV-1 significantly less effectively compared with chitin or wild type cells whilst binding HIV-1 with the same intensity.

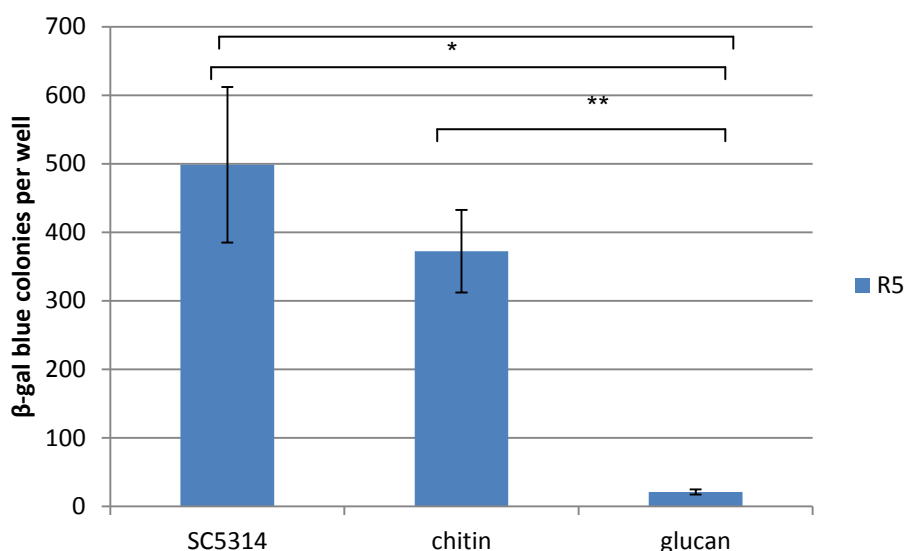


Figure 8-10: *C. albicans* and insoluble glucose moieties mutants transfer of YU2-R5 virus to T2M-bl reporter cells. SC5314 MOI=145. The same number of virus particles were incubated with similar volume insoluble moieties. Error bars represent standard deviation between wells (12). * and ** $p < 0.001$ by Student T-test. Data representative of 2 independent experiments.

8.4 Discussion

The presence of co-pathogens at mucosal surfaces may promote HIV-1 infection either through facilitating the exposure of HIV-1 to permissive immune cells in the epithelium (Klotman et al., 2008) or by inducing inflammation leading to activation of the HIV-LTR (Ferreira et al., 2011). Although epithelial cells are known to bind HIV-1 and transfer virus to permissive immune cells (Asin et al., 2004; Asin et al., 2003; Vacharaksa et al., 2008; Wu et al., 2003), as also demonstrated in Chapter 6, whether co-pathogens can also bind HIV-1 and transfer virus is largely unknown. *C. albicans* infections are common oral manifestations of HIV-1 infection, and as such there appears to be a link between HIV-1 and *C. albicans* infection (Flint and American Society for Microbiology., 2009). Some studies have shown that *C. albicans* can bind to certain HIV-1 components (Gruber et al., 2001; Gruber et al., 2003; Gruber et al., 1998; Wurznner et al., 1997) but the data are scarce. In this chapter we investigated this interaction further and find that while *C. albicans* does not alter HIV-1 receptor gene expression in oral and vaginal epithelial cells at the tested MOI, *C. albicans* does bind HIV-1 and is able to transfer infectious virus to permissive cells. Furthermore, we provide evidence that chitin and β -glucan (but probably not mannoproteins) are the fungal cell wall components responsible for HIV-1 binding, but that only chitin appears to permit viral transfer to susceptible cells.

All the *Candida* and *S. cerevisiae* species tested were able to bind HIV-1 X4 and R5 virus as determined by p24 detection using Western blotting. These data are partially consistent with the HIV-1 gp41 peptide binding ELISA data previously determined, which showed that while 2 types of *C. albicans* and other species (*C. dubliniensis* and 2 types *C. tropicalis*) were able to bind gp41, but that *C. krusei*, *C. glabrata* and *S. cerevisiae* were unable to bind gp41 (Gruber et al., 2003). We additionally showed that *C. guilliermondii* can also bind HIV-1. This fungal-viral interaction is notable and is likely to occur at mucosal surfaces when these fungal species are exposed to HIV-1.

Our data indicated that binding of HIV-1 to *Candida* would be independent of hypha formation, given that of the fungal species tested, only *C. albicans* and *C. dubliniensis* form true hyphae. This was confirmed using *C. albicans* mutants locked in the yeast ($\Delta eed1$), pseudohyphal ($\Delta tup1$) and hyphal ($\Delta nrg1$) forms, all of which were able to bind to HIV-1 R5 virus as determined by the p24 Western analysis. This is in contrast to previous findings that suggested the yeast form of *C. albicans* binds more poorly to HIV-1 gp41 than hyphae using the ELISA assay (Gruber et al., 2003). The differences between our data and that of Gruber et al (2003) most likely lie in the different assay used to investigate *Candida*-HIV-1 interactions (p24 Western versus gp41 peptide ELISA).

Given that all the fungal species tested were able to bind HIV-1 and that this was independent of morphology, we hypothesised that common cell wall structures (chitin, β -glucan and mannoproteins) shared by all fungal species are the likely components mediating viral binding. Using a series of glycosylation mutants defective in N- and O-glycosylation and purified insoluble chitin and β -glucan particles, we found that chitin and β -glucan appear to be the main fungal binding factors for HIV-1. Notably, we found no role for N- and O-glycosylation of proteins, which is in line with the supposition of Wurzner *et al.* (Wurzner et al., 1997), even though they did not directly test their supposition experimentally. However, chitin and β -glucan are present in all fungal species, and since HIV-1 appears to bind *C. albicans* more strongly than other *Candida* species, this either indicates that more chitin and β -glucan is exposed in *C. albicans* than in other *Candida* species or that other fungal factors also contribute to HIV-1 binding.

Of major interest was that *C. albicans* was able to transfer HIV-1 to permissive TZM-bl cells and that this might be mediated by chitin. Although the ability of other *Candida* species and *S. cerevisiae* to transfer HIV-1 to permissive cells was not investigated, we assume they would be able to transfer HIV-1 as chitin is present in the cell walls of all these fungal species. Of even greater interest was that HIV-1 was not transferred (or transfer greatly reduced) to TZM-bl cells from β -glucan. These intriguing

data may suggest that β -glucan binds to HIV-1 in a specific manner that may alter the HIV-1 envelope (gp41/120) structure so that it becomes inaccessible to canonical receptors (e.g. CD4, CCR5, CXCR4) on susceptible cells. It appears that glucan in complex with melanin (Seniuk et al., 2011) or as glucan sulphate (Tao et al., 2011a; Tao et al., 2011b), has potential as an anti-infective against HIV and bacteria (Mantovani et al., 2008). Thus, this highly interesting property indicates that β -glucan could act potentially as a HIV-1 microbicide by preventing transfer of immobilized HIV-1 on mucosal surfaces to immune cells in the sub-mucosa. However, further characterization of these findings is clearly required.

It should also be noted that β -glucans are polysaccharides of D-glucose monomers. Thus, interactions between HIV-1 gp41/gp120 and glucose residues present in the *C. albicans* cell wall may also explain the resistance of immobilized HIV-1 to removal from epithelial cells by trypsin digestion (which targets protein-protein interactions) as determined in Chapter 4, especially by X4 virus. Although speculative, this provides an additional potential mechanism for why R5 rather than X4 virus is preferentially transmitted across mucosal tissues, in that specific retention of X4 virus by β -glucan/glucose moieties in resident microbiota and mucosal secretions would prevent HIV-1 transfer to permissive cells in the sub-mucosa. Unfortunately, due to time constraints, we did not test whether X4 virus was retained more avidly or efficiently than R5 virus.

Very recently, a study identified a *C. albicans* gene, *HGT1*, which encodes the high-affinity glucose transporter 1 (Hgt1p) that may mediate binding to HIV-gp160 (Lesiak-Markowicz et al., 2011). However, the study was unconvincing and based only on the binding of the $\Delta hgt1$ mutant to a HIV-1 gp160 peptide as determined by ELISA. Also, gp160 binding was not eliminated in the $\Delta hgt1$ mutant and in fact was only reduced by less than 30%. Purified Hgt1 protein was not investigated and would have provided more definitive data regarding HIV-1 binding. Given that we found strong HIV-1 binding to all the fungal species tested as well as chitin and β -glucan (common to all fungi), it is unlikely that HIV-1 would target a single, specific protein for binding to *C. albicans*. Although this study was published after the experimental work of this thesis was completed, it would have been very interesting to test the $\Delta hgt1$ strain, together with the parent and revertant strains, in our assay systems to determine their HIV-1 binding capacity and ability to transfer infectious virus to permissive cells.

In order to take this work forward, β -glucan and chitin could be masked by blocking antibodies in order to determine if this would affect p24 binding to these moieties and transfer to susceptible cells. Also β -glucan linkages can be β 1-3 or β 1-6,

therefore these moieties could be inhibited separately to determine which is required for HIV-1 binding and the inhibition of transfer. In addition, the TZM-bl cells could be substituted for primary myeloid cells to verify the β -glucan data.

An experimental analysis of how HIV could “piggyback” on *C. albicans* hyphae that are invading mucosal tissues to infect susceptible cells was not considered. One could perhaps attempt this in primary explants or multilayered reconstituted epithelial models that would then be infected with fluorescently-labelled HIV and *Candida* and analysed using immunohistochemistry, FISH and/or primer-probe PCR for HIV integration in the epithelium.

8.5 Conclusion

HIV-1 interactions with *Candida* were characterised in this chapter. All fungal species tested (*C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. guilliermondii* and *S. cerevisiae*) were shown to bind HIV-1 R5 and X4 after 24 h and R5 after 2h exposure as determined by p24 Western analysis. HIV-1 binding was independent of (yeast or hyphal) morphology and appeared to be mediated by chitin and β -glucan in the cell wall, but not by N- and O-glycosylation structures of surface proteins. Notably, *C. albicans* was able to transfer infectious HIV-1 to susceptible reporter TZM-bl cells and this might be mediated by chitin. Of specific interest was that binding of HIV-1 by β -glucan may prevent transfer of HIV-1 to permissive cells, which identifies β -glucan as a potential microbicide to prevent mucosal HIV-1 transmission.

Chapter 9 Conclusion

The HIV pandemic has affected well over 40 million people worldwide since it was first identified thirty years ago. Like all viruses, HIV depends heavily on host-cell factors that enable the virus to enter cells, manifest the infection and produce progeny virions. Information regarding the interactions of HIV-1 with epithelial surfaces of the human oral and vaginal mucosa is limited in comparison with immune cells, and the exact cellular and molecular events involved in initial HIV-1 entry and mucosal infection are poorly understood. HIV-1 may use various strategies to infect the human host via mucosal surfaces and its transmission probably involves common steps, including survival of HIV-1 in mucosal secretions, interaction of HIV-1 with epithelial cells, transport/transmigration of the virus across the epithelium, and infection of CD4⁺/chemokine receptor⁺ target cells in the sub mucosa. In addition to these HIV-1-epithelial based events, disease progression in HIV-1 positive individuals is thought to be more rapid in the presence of co-pathogens, reducing survival and increasing the risk of HIV-1 transmission. These co-pathogens may include *C. albicans*, which is the most common fungal pathogen of humans and accounts for more than 50% of all fungal infections. Therefore, this project set out to investigate several aspects of HIV-1-mucosal interactions. The three main aspects of the work were:

1. To determine whether HIV-1 binding, integration and productive infection occurs in oral and vaginal epithelial cells.
2. To assess epithelial responses to HIV-1.
3. To characterise HIV-1 and *C. albicans* interactions.

9.1 HIV-1 binding, integration and productive infection in oral and vaginal epithelial cells

Using flow cytometry and Western blotting to detect gp120 and p24, we demonstrated that oral (TR146, FaDu) and vaginal (A431) epithelial cell models do not express discernable levels of CD4, CCR5 or CXCR4, but they do bind both X4 and R5 virus probably via non-canonical receptors. Once captured, HIV-1 remains infectious and can be transferred to permissive TZM-bl reporter cells. However, HIV-1 integration as measured by a primer-probe PCR was not detected. We conclude that while productive HIV-1 infection is unlikely to occur in epithelial cells under normal conditions (e.g. in health), epithelial cells may provide a potential route to establish systemic infection through sequestration and transfer of HIV-1.

One must consider the alternate view that healthy epithelia are quite effective barriers to HIV transmission (Greenhead et al., 2000). In many places they are multilayered, and the mucosae where they reside are hostile places for viruses to exist and remain infectious, let alone be transmitted. Since epithelial cells do not normally appear to get HIV-infected, they may play a passive role in retaining virus but the primary routes of transfer through them may only be by abrasion, or in the context of a co-infection causing inflammatory damage, or loosening of tight junctions that allow the virus to reach scavenging susceptible cells such as macrophages and dendritic cells in the mucosa (Greenhead et al., 2000; Shattock et al., 2003).

One of the key findings was that X4 virus binding to both oral and vaginal epithelial cells was more resistant to trypsin than R5 virus binding. This indicates that X4 viruses may predominantly utilise non-protein moieties (such as GalCer or carbohydrate/lectin-based molecules) to bind epithelial cells and R5 viruses may predominantly utilise protein moieties (such as HSPG's or ICAM-1). However, during the course of this write-up, a study also implicated mannose receptor in HIV-1 binding to vaginal epithelial cells (Fanibunda et al., 2011). It would, therefore, be interesting to determine a role for mannose receptor in mediating HIV-1 binding to and transfer from epithelial cells, perhaps using blocking antibodies or mannose to inhibit viral binding to the mannose receptor. Given that HIV-1 capture by epithelial cells may be a key first step in viral transfer to permissive immune cells, identification and manipulation of the non-canonical viral-specific receptors that mediate HIV-1 binding may have the potential to prevent primary systemic infections. As such, this should be a focus of future work. Furthermore, since oral, oro-pharyngeal and vaginal epithelial lines are able to transfer infectious virus to permissive cells, the next logical step would be to quantify the amount of transfer that occurs from these different epithelial cell lines. This would provide an indication of the efficiency of transfer and the subsequent relevance of this for infection *in vivo*. Also, blocking of receptors with small molecule inhibitors or antibodies should be prioritised in order to identify which impair transfer of HIV from epithelium to permissive cells.

An additional issue which is rarely discussed is that, currently, the consensus is that HIV-1 binding to epithelium is only mediated by HIV-1 gp120 interactions with receptors on the epithelial surface (Yahi et al., 1992; Yahi et al., 1995). However, when new viral progeny are produced they incorporate the host membrane during budding (Cantin et al., 2005; Paquette et al., 1998) as well as only approximately 14 virus spikes per particle (Zhu et al., 2006). Thus, the majority of the proteins on the viral outer surface (membrane) are host derived. As such, it is possible that these host proteins also play a role in the attachment of HIV-1 to epithelial cells rather than, or in concert with, HIV-1

gp120. To verify whether gp120 alone is required for viral-epithelial interactions, future experiments should expose epithelium to envelope (gp120)-deficient clones of HIV-1. This would allow us to determine whether HIV-1 binding of epithelium can be gp120 independent.

It is currently unknown how HIV-1 remains infectious on the epithelium (Dezzutti et al., 2001; Wu et al., 2003). One possibility is that HIV-1 gets taken into the epithelial cell by endocytosis and is retained in vesicles inside the cell prior to transfer of virus during interactions with permissive cells. Alternatively, HIV-1 may get enwrapped in microvillus structures on the surface of the epithelium protecting the virus from the harsh external environment. Data from our group (but not part of this thesis) demonstrates that when HIV-1 is pseudotyped with VSV-G envelope and therefore enters epithelial cells via the endocytic pathway, a productive HIV-1 infection ensues (Kohli *et al.*, 2012 submitted). This demonstrates two key points. First, that epithelial cells possess the cellular machinery to assemble and secrete infectious viral progeny if receptor-mediated entry is by-passed. Second, that HIV-1 does not normally enter epithelial cells via the endocytic pathway, otherwise a productive infection would ensue with live virus. These data would suggest that, post-capture, HIV-1 remains on the epithelial surface (possibly enwrapped in microvillus structures) and that viral transfer occurs from the surface. However, these possibilities would require further detailed investigations.

9.2 Epithelial responses to HIV-1

Since HIV-1 was able to bind to epithelial cells, albeit not resulting in a productive infection, we next attempted to unravel the responses of oral (TR146) and vaginal (A431) epithelial cell lines to HIV-1 exposure and determine the underlying signalling events. Although, HIV-1 gp120s and trimeric gp140s did not induce the tested cytokines in oral or vaginal epithelial lines, epithelium does appear to recognise whole virus particles. In TR146 cells, YU2-R5 virus activates the p38 pathway whereas LAI-X4 virus may inhibit p38, ERK1/2 and NF- κ B signalling. In A431, however, YU2-R5 virus activates the three main branches of the MAPK pathway (p38, JNK and ERK1/2) as well as IRF3, whereas LAI-X4 virus only activates p-38. Therefore, oral and vaginal cells appear to respond differently to HIV-1 R5 and X4 virus. A greater exploration of signalling pathways is warranted looking at TNF, IRF7 and IFN-induced genes as well as other intracellular signalling pathways. Notably, exposure of epithelial cells to HIV-1 R5 and X4 virus also induces cellular damage. Exploring the activation of various apoptotic pathways would be desirable in order to determine the origin of cellular damage. The role of these individual signalling pathways in mediating HIV-1 responses and damage is currently

unknown, but future experiments using either small chemical inhibitors or siRNA's would be informative in identifying the essential processes involved in the epithelial response to HIV-1. Furthermore, it would be valuable to confirm these findings in primary oral and vaginal epithelium.

The differences in signalling response to HIV-1 were confirmed in oral epithelial cells (primary and carcinoma) at the gene expression level by preliminary singleton microarray analysis. YU2-R5 increased apoptosis and cell death pathways and regulated the cell cycle while promoting transcriptional repression and cytokine activity. YU2-R5 also decreased negative regulation of apoptosis, regulation of cell adhesion and integrin mediated signalling. Conversely, LAI-X4 increased negative regulation of MAPK signalling, transcription and cell cycle regulation. Along with the previous data, this implies that oral epithelium is not an inert barrier to HIV-1 infection but instead responds to virus challenge, with similar pathways activated as those previously reported in immune cells. Given that these data were based on singleton arrays with oral epithelial cells, additional microarray investigation of both oral and vaginal epithelium with triplicate and properly controlled biological repeats would be highly informative in determining how epithelium responds to HIV-1 on a global scale. This would also possibly provide insights into host mediated events that may promote R5 over X4 virus selectivity during initial infection.

The potential importance of immune-regulation in HIV-1 transmission was recently indicated in study of HIV-exposed seronegative sex workers, which correlated high levels of anti-proteases in the vaginal mucosa with lower levels IL-1 α , monokine induced by interferon- γ (MIG) and interferon- γ -induced protein 10 (IP-10) in vaginal cervicovaginal lavages (Lajoie et al., 2012). These women also had limited T-cell inflammation in the mucosa as compared with other commercial sex workers and HIV-positive commercial sex workers. Overall the data lend support to the idea that immune quiescence may protect against systemic HIV-1 infection, which could be explored in future investigations.

9.3 Characterisation of HIV-1 and *C. albicans* interactions

Finally, we investigated the interaction of HIV-1 with human opportunistic pathogens in the genus *Candida*, predominantly *C. albicans*. All *Candida* species and *C. albicans* yeast and hyphae were able to bind and transfer HIV-1 to permissive cells. Notably, we identified two *C. albicans* cell wall molecules, chitin and β -glucan, that were capable of binding HIV-1. Neither has previously been implicated in interactions with HIV-1. Perhaps more importantly we found that HIV-1 could only be transferred to

permissive cells from chitin but not β -glucan, indicating that β -glucan may be able to sequester HIV-1 and thereby prevent primary infections. As β -glucan does not induce cytokine secretion or inflammation in epithelial cells (Moyes et al., 2010), β -glucan has considerable potential in prophylactic treatment and therapeutics to prevent HIV-1 infection. Thus, further investigations in this area are highly warranted.

Very recently, a study identified a *C. albicans* gene, *HGT1*, which encodes the high-affinity glucose transporter 1 (Hgt1p) that may mediate binding to HIV-gp160 (Lesiak-Markowicz et al., 2011). Given that we found strong HIV-1 binding to all the fungal species as well as chitin and β -glucan (common to all fungi), it is unlikely that HIV-1 would target a single, specific protein for binding to *C. albicans*. However, since HIV-1 binding to epithelial cells is trypsin sensitive (and thus, in part, protein mediated), especially R5 virus, it might also be useful to determine what proportion of HIV-1 binding to *Candida* is protein dependent and independent in future experiments.

As mentioned above, our group has discovered that HIV-1 is able to integrate into the epithelial genome when it enters via endocytic mechanisms (Kohli *et al* 2012 submitted). Although viral entry via endocytosis does not appear to occur under normal conditions (e.g. in health), it would be very interesting to test whether under inflammatory conditions, such as those caused by co-infections with secondary pathogens, HIV-1 could integrate into the epithelial genome. Since HIV-1 can directly bind *C. albicans* hyphae and the hyphal form is essential for epithelial invasion and inflammation, future studies investigating whether HIV-1 can 'piggy-back' on *C. albicans* hyphae to gain entry into epithelial cells would be of considerable interest. This would enable HIV-1 to by-pass conventional receptor-mediated mechanisms to gain entry into epithelial cells and integrate into the genome, thereby establishing a productive infection in mucosal tissues. A similar process of HIV-1 transmission may also occur with other oral and vaginal microbiota such as *Lactobacillus* species (vaginal) and *Streptococcus* species (oral). However, no data on this currently exists and such a hypothesis would need thorough investigation.

Lastly, recent studies using murine models suggest that viruses may take advantage of immune tolerance to local microbiota in order to evade the immune response and to promote infection (David, 2011; Kane et al., 2011; Kuss et al., 2011). One group used the mouse mammary tumour virus (MMTV), which is passed from mother to offspring, to show that when the mice were treated with antibiotics to eliminate gut bacteria, the pups could mount an effective immune response and clear the virus (Kane et al., 2011). Using knockout mice the authors implicated TLR4, its co-receptor CD14, IL-6 and IL-10 in mediating MMTV transmission and suggest the following sequence of events: TLR4/CD14 binds bacterial LPS and activates signal pathways that

results in IL-6 production which in turn induces IL-10 secretion. IL-10 dampens the immune response, thereby allowing MMTV to establish a chronic infection. Another group investigated the transmission of poliovirus and also found that gut microbiota had a role to play in viral transmission, as antibiotic treated mice demonstrated better survival rates than control animals following oral polio infection (Kuss et al., 2011). Polio also replicated less efficiently in the intestines of antibiotic treated mice versus mice that had not received antibiotics. The authors found that interaction of poliovirus with LPS increased viral infectivity. Thus, a picture appears to be emerging in the literature whereby mucosal viruses may potentially hijack the immune system by utilizing the tolerance mechanism developed to resident microbiota. This mechanism could also apply to other mucosally transmitted viruses such as HIV-1 and should be investigated in future experiments. For example, using the humanized mouse models for HIV-1 infection, RAG-hu (Berges et al., 2008), or the humanized bone marrow/liver/thymus mice (Mekus et al., 2006) one could pre-treat mice with antibiotics and identify whether the mice can clear HIV-1 upon vaginal and rectal challenge with X4 and R5 virus. The latter mouse model, where human fetal liver and human fetal thymus are implanted into irradiated NOD-scid IL2 γ ^{null}, develop systemic human immune cell types monocytes, macrophages and dendritic cells as well as T and B cell responses to HIV (Brainard et al., 2009). Although many virologists are not convinced by small animal models of human immunity, others are hopeful that they will help further our understanding of HIV transmission and biology (Singh et al., 2012; Nischang et al., 2012; Chang et al., 2012). If successful, undertaking similar experiments in new world monkey's challenged with SIV would be of interest.

In summary, microbes clearly interact with one another at mucosal surfaces in health and disease to generate different responses from the host. These interactions can improve an individual microbe's ability to colonise, invade or induce pathology. The breadth of these interactions can lead to difficulties in elucidating the microbial mechanisms used in the pathology of a disease, especially when these microbes are investigated in isolation from the wider microbiota or their potential partners. We must, therefore, continue to unravel this ever more complicated, intricate puzzle to gain a fuller understanding of events at mucosal surfaces and thus identify more effective therapies for the treatment and prevention of these infections.

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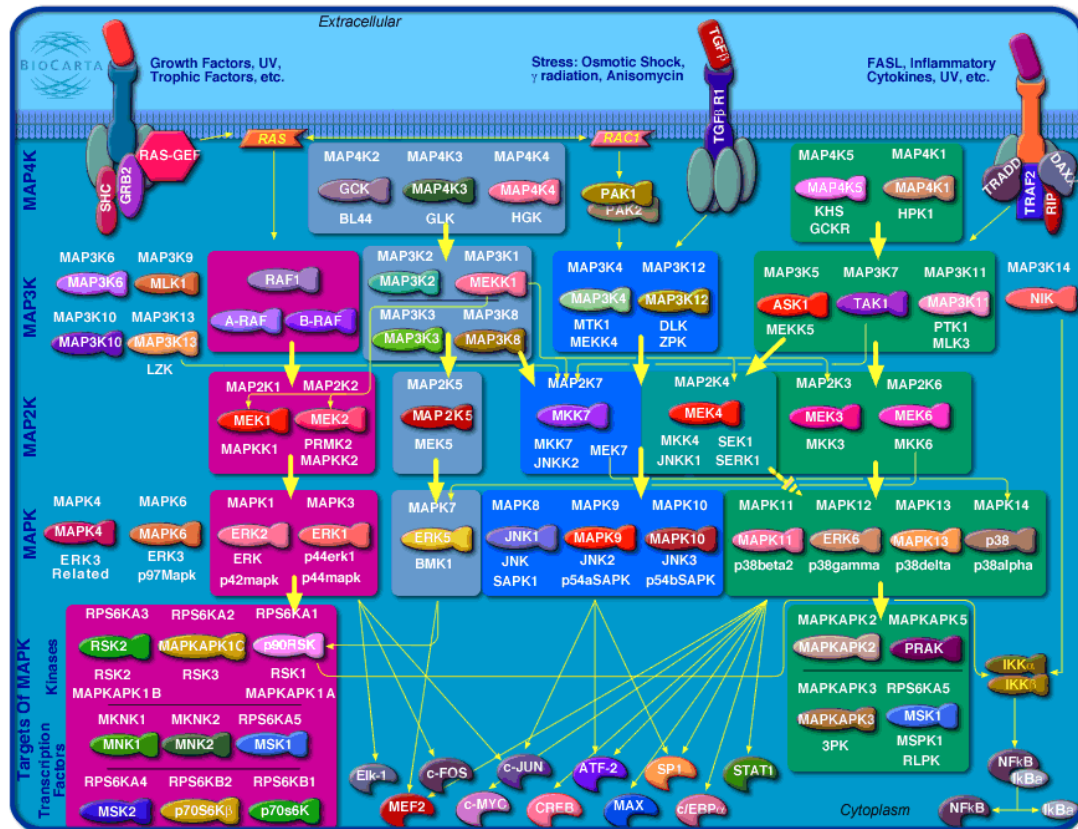
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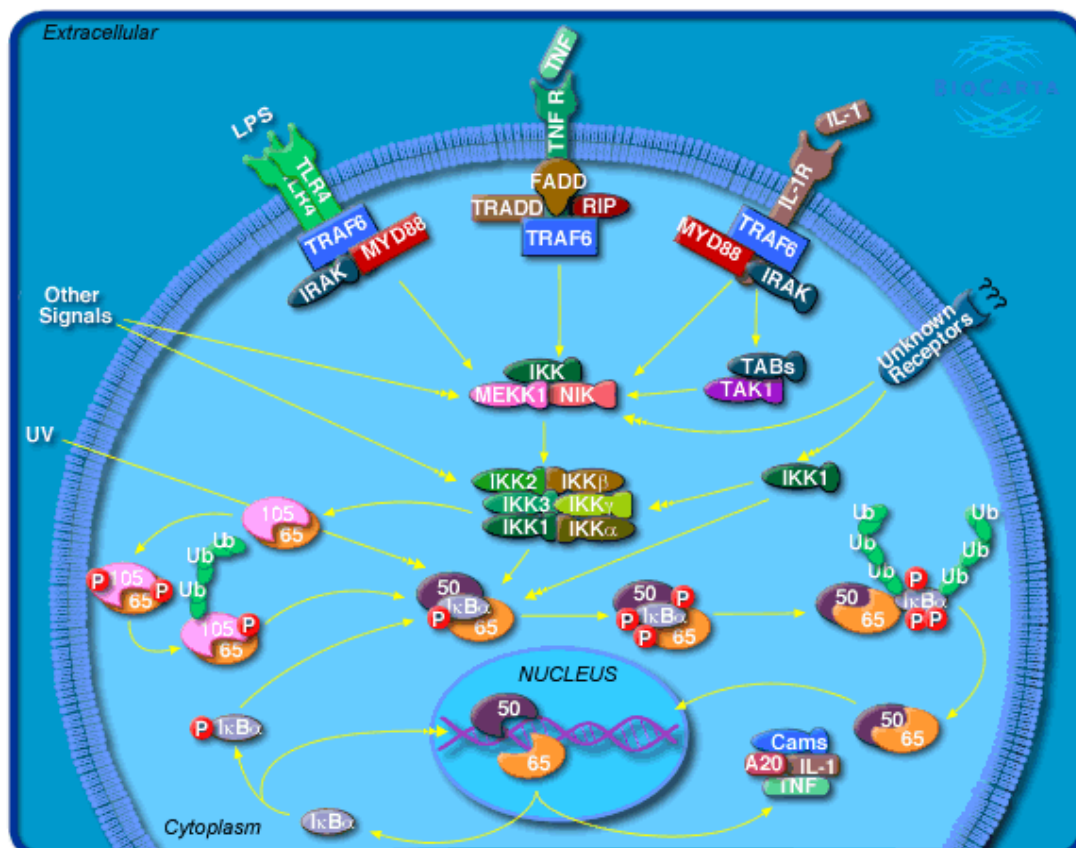
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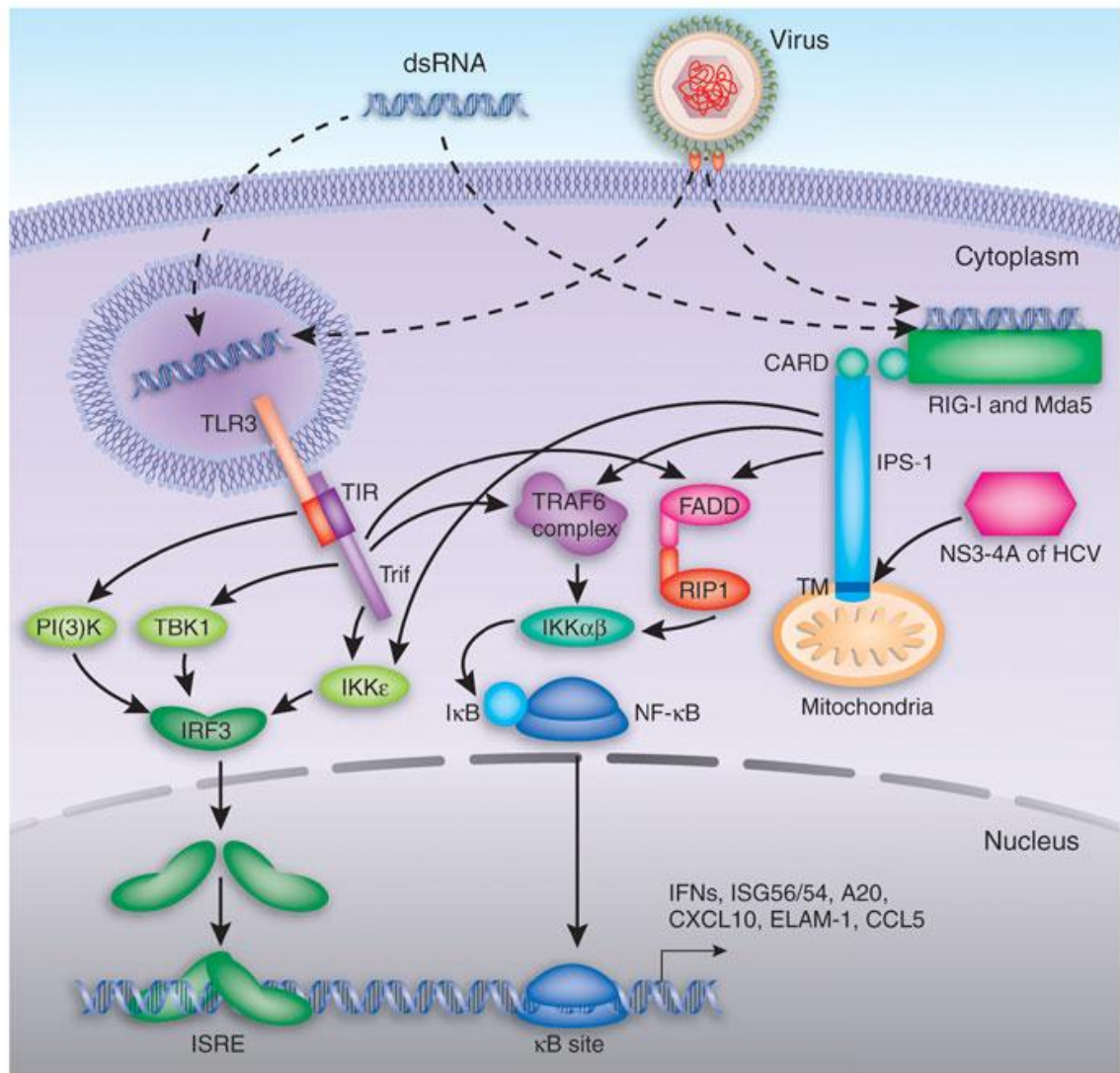
Appendix A: Pathway Diagrams



http://www.biocarta.com/pathfiles/h_mapkPathway.asp



http://www.biocarta.com/pathfiles/h_nfkbPathway.asp



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Appendix B: Submitted Manuscripts

Appendix C: Gp120 antibodies tried in flow cytometry

NIH#	isotype	origin	clade	storage	Concentration	Dilution for flow	Secondary	Notes
7369	IgG1, λ monoclonal	Human	Many	-70°C	43.6 μ g/ml	0.5-1 μ g/ml 2.3 μl/100 μl	Anti-human	Depends on tertiary protein structure, intact carbohydrate
2343	IgG2a	mouse	Many MN, IIB, RF	Not specified Now -70°C	unknown	1/50 2 μl/100 μl	Anti mouse	Against 1 st 204 aa of gp120. in some assays mediate ADCC
7624	IgG1, κ F425 a1g8	Human?	?	4°C	100 μ g total 2 μ g/ μ l in PBS	10-20 μ g/ml 2 μl/100 μl	Anti-human	CD4i epitope of gp120, enhanced by sCD4
7371	IgG1, κ	human	75% B, B', C, D	-70°C	149.3 μ g/ml 74.65 μ g total	0.5-1 μ g/ml 0.67 μl/100 μl	Anti-human	V2 region gp120, 164-194 gp120 HXB2
2640	IgG1, κ	Human ab in CHO cells	IIIB	-70°C	100 μ g at 1.4 μ g/ μ l	0.2-1 μ g/ml in neutralisation 0.71 μl/100 μl	Anti-human	Fab fragment against IIIB gp120 from long term asymptomatic seropositive.
1476	IgG1, κ	Human Monoclonal ab in CHO cells	IIB RF Broad range weakly MN, SF2	4°C	1 μ g/ μ l 250 μ l total			
7626	IgG2, κ			4°C	1.5 μ g/ μ l 100 μ l total	1 μl/100 μl 1-2 μ g		

						/100ul for flow		
810		murine	LAI				Anti- mouse	Aa 94- 97 LAI ELISA and western

Oral epithelial cells bind and transfer infectious HIV-1 to permissive cells but are not productively infected

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Full Title:	Oral epithelial cells bind and transfer infectious HIV-1 to permissive cells but are not productively infected
Short Title:	Oral epithelial cells bind and transfer HIV-1
Corresponding Author:	Julian R Naglik King's College London Dental Institute London, UNITED KINGDOM
Keywords:	HIV-1, epithelial, epithelium, mucosal, oral, expression, receptor, integration, infection, flow cytometry
Abstract:	Whether the oral mucosa can act as a portal of entry for HIV-1 infection remains a controversial issue. Given the fundamental importance such entry may have in the spread of HIV, there are relatively few publications in this area. To address this issue we utilized two epithelial cell lines representative of buccal (TR146) and pharyngeal (FaDu) sites of the oral cavity to determine (i) HIV-1 receptor gene and protein expression, (ii) whether HIV-1 integration into oral epithelial cells occurs, (iii) whether productive viral infection ensues, and (iv) whether infectious virus can be transferred to permissive cells. Using flow cytometry to measure captured virus by HIV-1 gp120 protein detection and western blot to detect HIV-1 p24 gag protein, we demonstrate that both buccal and pharyngeal epithelial cells capture X4 and R5 virus, probably via non-canonical receptors and are able to transfer infectious virus to permissive cells. However, HIV-1 integration, as measured by real-time PCR and presence of early gene mRNA transcripts and de novo protein production were not detected in either epithelial cell type. Our data demonstrate that although productive HIV-1 infection of oral epithelial cells leading to progeny virion production and viral dissemination is unlikely, it may provide a potential route as a mediator of systemic infection through attachment and transfer of HIV-1 to permissive cells.
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To PLoS ONE,

Dear Editor,

Please consider the enclosed manuscript entitled "Oral epithelial cells bind and transfer infectious HIV-1 to permissive cells but are not productively infected" for publication in PLoS ONE.

The majority of HIV-1 infections worldwide are acquired via mucosal surfaces, predominantly across the female or male genital tracts. Currently, the oral epithelium is generally recognized as a barrier to HIV-1 transmission. However, given the ambiguous literature on this fundamentally important topic, we aimed to determine whether HIV-1 binds, enters and integrates into oral epithelial cells, whether productive infection ensues, and whether sequestered infectious virus can be transferred to permissive cells. We utilized epithelial cell lines representative of two oral sites that are rarely investigated with regard to oral transmission but are one of the first cell types that are likely to come into contact with HIV-1 in the oral cavity. Buccal (TR146) and pharyngeal (FaDu) epithelial cells, both of stratified squamous cell origin, were utilized to permit convenient and direct comparisons to be made with regard to HIV-1 life cycle events.

Our extensive data demonstrate that HIV-1 X4 and R5 virus can readily bind oral epithelial cells (via non-canonical viral-specific receptors) but integration of the viral genome into epithelial cell DNA and *de novo* virus production were not detected. Thus, productive infection does not occur in oral epithelial cells. However, oral epithelial cells possess the cellular machinery to support productive HIV-1 infection if the virus enters via the endocytic pathway or if conventional entry mechanisms are by-passed. Once captured, HIV-1 also remains infectious on the surface of oral epithelial cells, which has important implications *in vivo* given the occurrence of viral transmission in nursing infants and during oro-genital contact in adults. The oral route may thus facilitate direct viral transfer to permissive cells in the sub-mucosa, thereby establishing acute infection by disseminating HIV-1 in the body. Therefore, although often considered a rare site of HIV-1 infection, the oral mucosa may have been overlooked as a potential route of viral transmission and mediator of systemic infection.

We would like to stress the novelty aspects of our work to PLoS ONE. First, TR146 and FaDu epithelial cell lines have not previously been used to investigate HIV-1 binding or infection. Thus, the entire data set is novel. Second, to our knowledge gp120 detection on the surface of epithelial cells and trypsin resistance to gp120 detection as measured by flow cytometry is also novel. Finally, the viral integration experiments using the qPCR based primer-probe method has not previously been used in oral epithelial studies. Finally, we stress that the research group that provided us with primary epithelial cells is no longer at KCL and so we can not perform any additional primary epithelial work.

Sincerely,

Dr Julian Naglik

Oral epithelial cells bind and transfer infectious HIV-1 to permissive cells but are not productively infected

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Running title: HIV-1 non-replicative infection in oral epithelial cells

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Abstract

Whether the oral mucosa can act as a portal of entry for HIV-1 infection remains a controversial issue. Given the fundamental importance such entry may have in the spread of HIV, there are relatively few publications in this area. To address this issue we utilized two epithelial cell lines representative of buccal (TR146) and pharyngeal (FaDu) sites of the oral cavity to determine (i) HIV-1 receptor gene and protein expression, (ii) whether HIV-1 integration into oral epithelial cells occurs, (iii) whether productive viral infection ensues, and (iv) whether infectious virus can be transferred to permissive cells. Using flow cytometry to measure captured virus by HIV-1 gp120 protein detection and western blot to detect HIV-1 p24 gag protein, we demonstrate that both buccal and pharyngeal epithelial cells capture X4 and R5 virus, probably via non-canonical receptors and are able to transfer infectious virus to permissive cells. However, HIV-1 integration, as measured by real-time PCR and presence of early gene mRNA transcripts and *de novo* protein production were not detected in either epithelial cell type. Our data demonstrate that although productive HIV-1 infection of oral epithelial cells leading to progeny virion production and viral dissemination is unlikely, it may provide a potential route as a mediator of systemic infection through attachment and transfer of HIV-1 to permissive cells.

Keywords

HIV-1, epithelial, epithelium, mucosal, oral, expression, receptor, integration, infection, flow cytometry

Introduction

The majority of HIV-1 infections worldwide are acquired via mucosal surfaces, predominantly across the female or male genital tracts [1]. In contrast, HIV-1 transmission through the oral mucosa is thought to be uncommon [2-7]. We and others have shown that several mechanisms may account for this, including neutralizing antibodies in seropositive individuals and innate anti-HIV inhibitory factors in saliva and/or epithelium [8-12]. However, studies in primates indicate that oral transmission is a possible occurrence since non-traumatic oral exposure to SIV results in regional dissemination followed by systemic infection [13-16]. Therefore, although oral epithelium may present a barrier to HIV-1 transmission, it may also be a conduit for viral entry. This is particularly important given the occurrence of viral transmission in nursing infants and during oro-genital contact in adults.

Entry of HIV-1 into permissive host cells requires expression of the receptor CD4 and a fusion co-receptor (chemokine receptors CCR5 (R5-tropic) or CXCR4 (X4-tropic)) [17]. However, the vast majority of reports indicate that oral epithelial cells do not express CD4 [18-22] and express CCR5 and CXCR4 at either undetectable or very low levels [11,18,21,23,24]. Despite these receptor dependencies, HIV-1 may also infect CD4⁺ cells such as spermatozoa [25], fibroblasts [26] and oligodendrocytes [27], as well as epithelial cells from other mucosal sites including the thymus [28], cervix [29] and the gastrointestinal tract [30]. Hence, HIV-1 may utilize several alternative receptor mechanisms for binding and entry into cells. Besides binding to canonical entry receptors, the viral envelope protein gp120 also binds to several other cell-surface molecules including DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) [31,32], GalCer (glycosphingolipid galactosylceramide) [27,33,34], and heparan sulphate proteoglycans (HSPGs) such as syndecan-1 [35,36]. GalCer and HSPGs are commonly expressed on epithelial cells and may promote HIV-1 binding [11,36,37]. Importantly, there is a preference for R5-tropic viral transmission across mucosal surfaces [38], but a full and satisfactory explanation for this has not yet been provided.

The fundamental issue of whether oral epithelial cells can be productively infected with HIV-

1 remains controversial. Although the recovery rate of infectious HIV-1 from saliva is low, proviral
DNA has been detected in oral epithelial cells [39]. Likewise, the presence of HIV-1 gag RNA has
been demonstrated in both buccal cells [40,41] and oral biopsies [41,42]. These findings suggest
that HIV-1 proviral DNA may integrate into the epithelial cell genome, but the literature remains
ambiguous as to whether a productive infection ensues. For example, using primary gingival
epithelial cells, one study showed susceptibility to R5 but not X4 tropic viral strains in a CD4-
independent manner [43], whilst another study showed X4 rather than R5 susceptibility [18], with
epithelial cells being able to secrete infectious virus. Primary epithelial cells isolated from adenoids,
palatine tonsils and salivary glands may also be productively infected with HIV-1 [43-48].
However, others have found no evidence of productive HIV-1 infection in oral epithelial cells
[20,21]. Rather, HIV-1 is preferentially sequestered and transferred to permissive cells to establish a
primary infection [21,36,49-52].

Given the ambiguous literature on this fundamentally important topic, we aimed to determine
whether HIV-1 binds, enters and integrates into oral epithelial cells, whether productive infection
ensues, and whether sequestered infectious virus can be transferred to permissive cells. We utilized
epithelial cell lines representative of two oral sites that are rarely investigated with regard to oral
transmission but are one of the first cell types that are likely to come into contact with HIV-1 in the
oral cavity. Buccal (TR146) and pharyngeal (FaDu) epithelial cells, both of stratified squamous cell
origin, were utilized to permit convenient and direct comparisons to be made with regard to HIV-1
life cycle events. We demonstrate that both epithelial cell types were able to capture X4 and R5
virus but integration of the viral genome into epithelial cell DNA and *de novo* virus production were
not detected. Notably, VSV-G-packaged HIV-1 was replication competent in both epithelial cell
lines. Finally, both epithelial cell types were able to transfer infectious virus to permissive cells,
which *in vivo* would permit infection of immune cells in the sub-mucosa and dissemination of HIV-
1 in the body.

Methods

Ethics Statement

Primary epithelial cells (gingival) were obtained from wisdom tooth extractions and were kindly provided by Maxine Partridge and collection approved by the Guy's Ethics Research Committee.

Cell lines, primary cells, viruses and virus-like particles

Human oral buccal (TR146) and pharyngeal (FaDu) carcinoma cell lines and 293T (renal epithelial) cells were obtained from the American Type Culture Collection. Human glioma cells (NP2) expressing human CD4 and CXCR4 or CCR5 have been previously described [53]. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl cells (catalogue no. 8129), PM1 cells (catalogue no. 3038), 8E5/LAV cells (catalogue no. 95), C8166 T cells (catalogue no. 404 donated by Robert Gallo), JTLRG-R5 (catalogue no. 11586) and HIV-1 molecular clones pYU2 (R5-utilizing, catalogue no.1350) and pLAI.2 (X4-utilizing, catalogue no. 2532). The HIV-1 *gag-pol* expression vector p8.91 and the vesicular stomatitis virus envelope protein (VSV-G) expression vector pMDG were kindly provided by Didier Trono (University of Geneva, Switzerland). The HIV gp160 envelope vectors (pHXB2 (X4), pYU2 (R5) and pSVIII 89.6 (dual tropic)) were a gift from Professor Greg Towers, University College London. The retroviral packaging vector pCSGW encoding GFP was a kind gift from Adrian Thrasher, Institute of Child Health, University College London, UK. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM, PAA) supplemented with 10% fetal bovine serum (FBS) (PAA), 100 U of penicillin per mL, and 100 µg of streptomycin per mL (PAA) at 37°C and 5% CO₂. NP2 cells were additionally supplemented with 1 mg/mL of G418 (Invitrogen) and 1 µg/mL of puromycin (Invitrogen).

Virus preparation

Viral vectors used for production of VSV-G and HIV gp160 pseudotyped HIV were prepared by transient transfection of 293T cells using a protocol adapted from Besnier *et al.* [54]. Briefly, 293T cells were seeded at 95% confluency in a 10 cm³ dish and the following day cells were transfected with 3 µg each of pMDG and p8.91 and 4.5 µg of pCSGW using the polyanionic transfection reagent Jet PEI (Polyplus Transfection) according to the manufacturer's instructions. After 24 h the media was replaced and 48, 72 and 96 h post-transfection virion-containing culture supernatants were harvested and filtrated through a 0.45µm pore size membrane and stored in aliquots at -80°C until required. Production of infectious stocks of live virus was performed by transient transfection of 293T cells as described above, with 5 µg of the infectious molecular clone pLAI.2 (X4) and or pYU2 (R5) used per transfection. For trypsin sensitivity experiments and detection of integration into epithelium, YU2 virus was grown in NP2-R5 and JLTRG-R5 cells while LAI virus was grown in C8166 cells for 1- 2 weeks with addition of fresh medium until cells showed cytopathic effects and were then harvested and frozen in aliquots.

Virus titration

Infectious virus stock (LAI and YU2) titers were determined by plaque assay. Briefly, TZM-bl cells (1×10^4 cells/well) were cultured overnight (96-well plates) and incubated with eight replicates of ten serial dilutions (0.5 log) of virus stock in a total of 100 µL growth media per well. After 48 h, virus supernatant was removed and the cells were fixed with 0.05% glutaraldehyde for 5 min at room temperature and washed twice with phosphate-buffered saline (PBS). Expression of β-galactosidase was determined by staining cells with X-Gal stain [1mg/mL X-Gal in 5 mM KFe₄(CN)₆ 3H₂O, 5 mM KFe₃(CN)₆ 3H₂O, and 1 mM MgCl₂] and incubating culture plates at 37°C for 2 h. Virus infectivity was estimated as plaque forming units (PFU) per mL. Titration of VSV-G pseudotyped HIV-1 and HIV gp160 pseudotyped HIV-1 was carried out using 293T cells or NP2 cells, respectively. Cells were seeded at 1×10^5 cells/well (24-well plates) and cultured overnight at

37°C. Serial dilutions (1:2) of virus supernatant were applied to the cells (500 µL) and incubated overnight. The following day the media was exchanged and 48 h after transduction with virion-containing culture supernatants the percentage of GFP-expressing cells was determined by flow cytometry using the FACSCanto machine (BD Biosciences). Data was analyzed with FACSDiva software and WinMDI (copyright 1993-2000 Joseph Trotter <http://facs.scripps.edu>) to calculate the infectious units per mL.

HIV-1 receptor expression by quantitative reverse transcription-PCR

RNA was isolated from resting TZM-bl, TR146 and FaDu cells using GenElute Mammalian Total RNA Miniprep Kit (Sigma), followed by treatment with Turbo DNA free DNase (Ambion) according to the manufacturer's instructions. All samples were confirmed DNA free prior to analysis. cDNA was synthesized from 1 µg of RNA using HIV reverse transcriptase (Ambion) according to the manufacturer's instructions. Primers were obtained from RTPPrimerDB (<http://medgen.ugent.be/rtprimerdb/>) and PrimerBank (<http://pga.mgh.harvard.edu/primerbank>) [55,56]. Gene expression of CD4, CCR5, CXCR4, DC-SIGN, SDC-1 (syndecan-1) and SDC-4 (syndecan-4) was quantified by real-time PCR using SYBR Green JumpStart Taq Ready Mix (Sigma) with 4 pmol primers and 1 µL cDNA in 10 µL reactions on the Corbett Research Rotor-Gene 6000 (Qiagen) using the following cycling parameters: 95°C for 3 min; followed by 95°C for 3 s, annealing for 10 s and extension for 20-30 s for 40 cycles. Data was analyzed with Corbett Research Rotor-Gene 6000 Series Software 1.7 using the two standard curve method with β-actin used as the normalizer gene. Primer sequences, annealing and extension temperatures are listed in Table 1.

HIV-1 receptor expression by flow cytometry

TZM-bl, NP2-X4, NP2-R5, TR146 and FaDu resting cells were washed with PBS and incubated with 0.02% (W/V) EDTA for 5-30 min. Detached cells were washed thoroughly with PBS

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supplemented with 1% BSA and 0.01% azide (wash buffer), and resuspended at 1×10^6 cells in 1
mL wash buffer. To identify surface expressed HIV-1 receptors and co-receptors, 100 μ L of cells
were incubated at room temperature for 1 h with mouse anti-human CD4 (1:4 catalogue no.724),
CCR5 (1:20, catalogue no. 4090), CXCR4 (1:80, catalogue no. 4083), DC-SIGN (1:100 catalogue
no. 6884) monoclonal antibodies (all obtained from the AIDS Research and Reference Reagent
Program), GalCer (1:200, anti-galactocerebroside, Millipore) or heparan sulfate proteoglycan
(1:200, Millipore) monoclonal antibodies. Primary antibodies were detected with goat anti-mouse
IgG conjugated with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch). After thorough
washing, cells were fixed in 200 μ L 4% formaldehyde and the percentage of FITC-expressing cells
was determined by flow cytometry.

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Detection of HIV-1 binding and replication by Western Blot

TR146, FaDu and TZM-bl cells were seeded at 5×10^5 cells per well and the following day infected
with YU2 (R5) or LAI (X4) virus at a multiplicity of infection (MOI) of 0.2. After overnight
incubation at 37°C the cells were washed to remove unbound virus. Cells were harvested in 250 μ L
1 x RIPA buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium
deoxycholate and 0.1% SDS, supplemented with Halt complete protease inhibitor cocktail (Perbio
Science)], placed on ice for 30 min, and stored at -80°C until required. Total protein lysates
(mammalian and viral) were normalized for protein content using the bicinchoninic acid (BCA)
assay (Pierce) and separated using 12% SDS-PAGE gels. Proteins were transferred to PVDF
membranes, probed with anti-HIV-1 gag monoclonal antibody recognizing p24 and p55 isoforms
(catalogue no.6457)) and secondary goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated
antibody (Jackson ImmunoResearch), before developing using Immobilon-ECL (Millipore). α -actin
was used as a loading control.

Detection of HIV-1 binding and packaged viral RNA by PCR

TR146, FaDu and TZM-bl cells were seeded at 5×10^5 cells per well and the following day infected with YU2 (R5) or LAI (X4) virus at an MOI of 0.2. After overnight incubation at 37°C the cells were washed to remove unbound virus. Total RNA was isolated as above and confirmed DNA free prior to analysis. Equal amounts of total RNA was used to detect packaged viral RNA by first synthesizing cDNA using Superscript cDNA Synthesis Kit (Invitrogen) and an HIV-1 specific primer (5'-GTC ATG AAA CAA ACT TGG C-3'). A 2 µL aliquot of cDNA was then subjected to nested PCR using primers to amplify a 2 kb fragment of the HIV *pol* gene. First round PCR was performed in a 20 µL reaction containing 1 x PCR buffer, 100 µM dNTP's, 1.5 mM MgSO₄, 2.5 U *Taq* polymerase (New England Biolabs) and 10 pmol of each primer (Forward: 5'-AAT GAT GAC AGC ATG TCA GGG AGT-3'; Reverse: 5'-AGT CTT TCC CCA TAT TAC TAT GCT TTC-3'). Cycle parameters were as follows: 95°C for 5 min; 94°C for 10 s, 55°C for 30 s, and 72°C for 1 min for 30 cycles; and an extension of 72°C for 10 min. For subsequent nested PCR, 1 µL of the first round PCR reaction was used as a template to amplify an internal region of the *pol* gene and was performed in a 10 µL reaction containing 1 x SYBR Green JumpStart Taq Ready Mix (Sigma), and 3 pmol of each primer (Forward: 5'-TTC TTC AGA GCA GAC CAG-3'; Reverse: 5'-ACT TTT GGG CCA TCC ATT-3'). Cycle parameters were 95°C for 3 min; followed by 95°C for 1 min, 55°C for 30 s, and 72°C for 1 min for 35 cycles; and an extension of 72°C for 10 min. PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

Whole virus binding and trypsin sensitivity

TZM-bl, TR146 and FaDu cells (5×10^4) were infected with either YU2 (R5) or LAI (X4) virus at an MOI of 5 overnight at 4°C. Cells were washed three times with PBS and blocked in PBS/10% BSA for 10 min at room temperature. To determine whether HIV-1 binding was trypsin sensitive, prior to blocking, cells were treated with trypsin (0.05%)-EDTA (0.02%) (PAA, UK) for 5 min at 37°C. Cells were gently removed by scraping and labeled with HIV-1 gp120 monoclonal antibody

F425 B4e8 (1:200) (AIDS Research and Reference Reagent Program) followed by Cy5-conjugated AffinityPure goat anti-human IgG secondary (1:400) (Jackson ImmunoResearch), each for 30 min at 4°C. Cells were washed three times with PBS, resuspended in 4% formaldehyde and subjected to flow cytometry. Binding percentages for whole virus were calculated as increased Cy5 shift of HIV-1 infected labelled cells from uninfected labelled cells. Given the higher background with a new batch of antibody, binding percentages for the trypsin sensitivity data were calculated as increased Cy5 shift relative to HIV-1 infected secondary alone labelled cells.

Detection of HIV-1 integration by primer-probe Alu-LTR PCR assay

To determine whether HIV DNA was able to integrate into epithelial cells a real-time PCR assay was performed with HIV-1 LTR and human Alu-specific primers with a U5 specific probe as previously described [57]. TR146, FaDu and PM-1 (control) cells were seeded at 5×10^5 cells per well and the following day infected with YU2 (R5) or LAI (X4) virus, pre-treated with RNase-free DNase (Roche, UK) at 37°C for 1 h with 4mM MgCl₂. MOI's ranged from 1 to 140. Heat-inactivated virus (60°C for 1 h) without DNase treatment was used as a DNA contamination control. Cells and virus were incubated for 48 h at 37°C, after which cells were washed three times with PBS before DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, Poole, UK) according to the manufacturer's instructions with Proteinase K digestion for 20 min. Isolated DNA samples were digested with DpnI (New England Biolabs, UK) to degrade any plasmid DNA contaminant. DNA was then quantified by Nanodrop and either 50 ng or 100 ng DNA was analysed by real-time PCR on a Rotorgene 6000 (Qiagen, UK) using primers 0.2 M MH535 forward (5'-AACTAGGGAACCCACTGCTTAAG-3') and 0.8 M reverse SB704 (5'-TGCTGGGATTACAGGCGTGAG-3') with 0.2 M probe P-HUS-SS1 (5'-FAM-TAGTGTGTGCCCCGTCTGTTGTGTGAC-TAMRA-3') using Jumpstart Ready Mix (Sigma, Poole, UK) in 10 µL reactions. For each PCR reaction the same concentration of DNA was used for all samples isolated from individual cell lines. Samples were denatured for 10 min followed by 60

cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 60 s. DNase-treated virus exposed samples were compared with heat-inactivated virus exposed samples. Positive integration events were taken as a lower C_t in the DNase treated virus exposed sample than the heat-inactivated virus control.

Productive viral infection by detection of spliced HIV-1 *tat* by PCR

TR146, FaDu and TZM-bl cells were seeded at 5 x 10⁵ cells per well and the following day infected with YU2 (R5) or LAI (X4) virus at an MOI of 0.2. After overnight incubation at 37°C unbound virus was removed by washing and total RNA isolated (GenElute Mammalian Total RNA Miniprep Kit; Sigma). Genomic DNA was removed with Turbo DNase free (Ambion) according to the manufacturer's instructions and samples were confirmed DNA free prior to analysis. Equal amounts of total RNA was used to synthesize viral cDNA transcripts using the HIV-specific oligo ART-7 5'-TTC TAT TCC TTC GGG CCT GTC G -3'. A 1 µL aliquot of cDNA was then subjected to PCR using primers spanning the *tat*1 and 2 exon junctions (*tat*-junction forward: 5'- TAG ATC CTA GAC TAG AGC CC-3' and *tat*-junction reverse 5'- TTG GGA GGT GGG TCT GAA ACG-3') in a 20 µL reaction containing 1 x PCR buffer, 100 µM dNTP's, 1.5 mM MgSO₄, 2.5 U *Taq* polymerase (New England Biolabs) and 10 pmol of each primer. Cycle parameters were as follows: 95°C for 5 min; followed by 94°C for 10 s, 55°C for 30 s, and 72°C for 1 min for 35 cycles; with a final extension of 72°C for 10 min. PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

HIV-1 integration and productive infection using pseudotyped virus-like particles

TR146, FaDu, NP2-R5 and NP2-X4 cells were seeded at 1 x 10⁵ cells per well and cultured overnight at 37°C. Serial dilutions (1:2) of HIV-1 gp160 pseudotyped (X4, R5 and dual tropic) and VSV-G pseudotyped HIV-1 were applied and incubated overnight at 37°C. HIV-1 integration and *de novo* virus protein production were determined by the presence of GFP-expressing cells by flow cytometry. To inhibit HIV-1 specific GFP expression, infections were also carried out in the

presence of 500 μ M of the HIV-1 reverse transcriptase inhibitor AZT (NIH AIDS Reagent Program Cat no.3485).

Detection of *de novo* HIV-1 production by indicator cell infection

TR146, FaDu, and TZM-bl cells were seeded at 5×10^5 cells per well and the following day infected with YU2 (R5) or LAI (X4) virus at an MOI of 0.2. After overnight incubation at 37°C the cells were extensively washed with HBSS (Invitrogen) to remove unbound virus. Fresh media was applied to the cells and the plates were incubated at 37°C for up to 7 days to allow any *de novo*-produced infectious virus to be released into the medium. Culture medium (potentially containing infectious virus) was then applied to 3×10^5 TZM-bl indicator cells and incubated for a further 24 h at 37°C. Cells were fixed, washed twice with PBS and stained for β -galactosidase expression with X-Gal stain. Individual wells were visualized by light microscopy at 100 X magnification.

HIV-1 transfer assay

FaDu, TR146 and TZM-bl cells were seeded at 1×10^5 cells and the following day infected with YU2 (R5) or LAI (X4) virus at an MOI of 0.2. After overnight incubation at 37°C the cells were thoroughly washed in HBSS to remove any unbound virus. Controls included FaDu and TR146 cells without the addition of virus. TZM-bl cells (3×10^5) were then overlaid onto the epithelial cells and the plates incubated for a further 48 h at 37°C. Cells were fixed and stained for β -galactosidase expression with X-Gal stain. Individual wells were photographed by light microscopy at 100 X magnification.

Results

Expression of HIV-1 receptors in oral epithelial cells

The initial step of HIV-1 infection of any cell type is surface receptor binding. Therefore, we first analyzed the gene expression levels of canonical (CD4, CCR5, CXCR4) and non-canonical (DC-

SIGN and HSPG's, excluding GalCer as this non-protein moiety can not be investigated by gene expression) HIV-1 receptors in oral epithelial cells by quantitative PCR (Fig. 1). This demonstrated the absence or minimal expression of CD4, CCR5 and CXCR4 in both TR146 and FaDu cells compared with control TZM-bl cells. In addition, in all three cell types DC-SIGN was minimally expressed and the HSPG syndecan-1 was highly expressed. The only major difference observed between the two oral epithelial cell types was the high expression of the HSPG syndecan-4 in FaDu cells (at levels greater than TZM-bl cells), which was undetectable in TR146 cells.

We next determined the surface expression of CD4, CCR5, CXCR4, DC-SIGN, GalCer, and HSPG's on oral epithelial cells by flow cytometry (Fig. 2). Both TR146 (buccal) and FaDu (pharyngeal) cell lines expressed undetectable levels of CD4 and low levels of CCR5 and CXCR4 (~6-8%) compared with control TZM-bl cells, which are HeLa cell derivatives engineered to express higher levels of CD4 (32%), CCR5 (17%) and CXCR4 (85%), supporting the gene expression data. NP2 cells expressing either CCR5 (85%) or CXCR4 (88%) were also used as positive controls and expressed high levels of CD4 (50-60%). With regard to non-canonical receptors, both TR146 and FaDu cells expressed similar amounts of DC-SIGN (6-8%) and HSPG's (6-10%) but FaDu cells expressed greater amounts of GalCer than TR146 cells (25% and 15%, respectively). Of note, the similar amounts of surface HSPG's expressed on TR146 and FaDu cells appears to be in contrast to the differences in gene expression data for syndecan-4 (Fig. 1); however, this was expected since the detecting antibody recognizes other HSPG's including syndecan-1, which is highly expressed in both TR146 and FaDu cells (Fig. 1). TZM-bl cells also expressed low levels of DC-SIGN (14%), GalCer (14%) and HSPG's (7%). Likewise NP2-X4 and -R5 cells expressed low levels of DC-SIGN (5-20%), but higher levels of GalCer (25-50%).

HIV-1 binding to oral epithelial cells

We next determined whether HIV-1 can be captured by both oral epithelial cell types. TR146, FaDu and TZM-bl cells were incubated overnight with cell free YU2 (R5) or LAI (X4) infectious virus.

After extensive washing the presence of attached virus was determined using three separate approaches.

First, total protein was isolated using RIPA buffer and the detection of HIV-1 p24 gag protein determined by immunoblot analysis. p24 was present in both TR146 and FaDu protein lysates at levels similar to that found with TZM-bl cells, indicating that both R5 and X4 virus are captured by both buccal and pharyngeal epithelial cells (Fig. 3A). We confirmed that R5 and X4 virus are also captured by primary oral epithelial cells (gingival) (Fig. 3A). Given that primary epithelial cells demonstrated identical HIV-1 binding data to TR146 and FaDu cells, all other experiments were performed with TR146 and FaDu cells only.

Second, using a more quantitative approach, the presence of immobilized virus on the surface of TR146 and FaDu cells was determined by flow cytometry using a Cy5-labeled anti-human secondary antibody to detect a monoclonal primary that detected HIV-1 gp120 F425 B4e8. Both R5 and X4 virus was detected on TR146 and FaDu cells demonstrating direct binding of infectious virus to both epithelial cell types (Fig. 3B). However, FaDu cells appeared to capture both R5 and X4 virus more efficiently than TR146 cells (27-32% versus 4-7%, respectively) and to a similar degree as control TZM-bl cells (21-59%). A second monoclonal primary F425 A1g8 antibody showed similar binding to the epithelial lines as F425 B4e8 (data not shown).

Third, we also determined the presence of captured virus through the detection of packaged HIV RNA by amplification of the HIV-1 *pol* gene using nested PCR. Given that this approach was used to confirm the p24 protein and whole virus binding data, we performed this experiment using R5 virus only. Successful amplification of the HIV-1 *pol* gene indicated the presence of R5 virus on both TR146 and FaDu cells in addition to TZM-bl cells (Fig. 3C). It should be noted that the Western blot (Fig. 3A) and PCR (Fig. 3C) data are qualitative and may not reflect differences in the efficiency of R5 and X4 HIV-1 binding to TR146 and FaDu cells, which was more apparent when using the quantitative flow cytometry approach (Fig. 3B).

Finally, we determined that binding of both R5 and X4 virus to TR146, FaDu and TZM-bl cells was trypsin sensitive, with between 73-97% of R5 and 33-78% of X4 virus being removed upon 5 min incubation with trypsin at 37°C (Fig. 3D). This suggests that R5 and X4 virus interact predominantly with protein moieties on epithelial cells to mediate binding but that X4 virus may also utilize additional non-protein moieties, as X4 binding was less sensitive than R5 binding to trypsin digestion to both TR146 and FaDu cells. Taken together, the data demonstrate that both R5 and X4 virus directly bind buccal and pharyngeal epithelial cells, with possible preferential binding to pharyngeal cells.

HIV-1 mRNA transcription and *de novo* viral protein production

Given that HIV-1 was able to bind both TR146 and FaDu cells, we hypothesized that epithelial cells may support productive infection resulting in HIV-1 mRNA transcription and *de novo* viral protein production. This would ultimately result in the assembly of new viral progeny, demonstrating that a productive infection has been established. To investigate this we utilized four different approaches.

First, we used a PCR-based system to detect spliced HIV-1 *tat* mRNA in the target epithelial cells 24 h post-infection with infectious R5 and X4 virus. The presence of high levels of spliced *tat* mRNA indicates HIV-1 integration and *de novo* production of viral mRNA transcripts, which in permissive cells is representative of a productive HIV-1 infection that results in new viral progeny being produced. We found that spliced *tat* mRNA was only detected in control TZM-bl cells but not in TR146 or FaDu cells (Fig. 4A).

Second, to determine if *de novo* virus production could be detected we probed protein lysates of TR146, FaDu and TZM-bl cells for p55 gag protein by Western blot 24 h post-infection with YU2 (R5) or LAI (X4) virus. Detection of p55 gag protein is only observed if *de novo* viral protein production has occurred. Fig. 4B shows p55 gag protein expression only in the control TZM-bl cells but not in the TR146 and FaDu cells oral epithelial cells.

Third, given that most analyses were undertaken 24 – 48 h post-infection with HIV-1, we

hypothesized that epithelial cells may require a longer time period for viral progeny to be produced. Therefore, after addition of infectious R5 and X4 virus to TR146 and FaDu cells for 24 h, the cells were thoroughly washed and the medium replenished for up to 7 days. Culture supernatants taken at day 4 and 7 (potentially containing new viral progeny) were then transferred onto TZM-bl indicator cells for 48 h. Any *de novo* virus production and subsequent infection of TZM-bl cells would cause HIV-LTR driven β -galactosidase production, which would be visible as blue foci in the assay. However, no blue foci were observed indicating that no new virions were produced after prolonged virus incubation in either epithelial cell type (data not shown).

Fourth, we utilized the three-plasmid expression system developed by Naldini *et al* [58] to generate HIV-based vectors pseudotyped with HIV-1 envelopes, HXB (X4), YU2 (R5) or 89.6 (dual tropic). Supernatants containing replication defective retroviral particles were used to transduce TR146, FaDu and NP2-R5/X4 cells. NP2 cells were used because the receptor expression is maintained using selective media and thus efficiency of viral infection was greater than with TZM-bl cells, so these cells were used as the positive control. Unlike the HIV canonical receptor expressing NP2 cells, TR146 and FaDu cells failed to drive expression of the GFP reporter gene resulting in undetectable GFP fluorescence up to 48 h post-infection (Fig. 4C). Taken together, our extensive data sets indicate that productive HIV-1 infection does not occur in TR146 and FaDu epithelial cells.

HIV-1 integration into epithelial cells

Although both R5 and X4 virus was unable to productively infect TR146 and FaDu cells, it was possible that, post-capture, HIV-1 was able to gain entry and integrated into the epithelial cell DNA to establish a latent infection. To test for this possibility we performed a real-time PCR assay to detect integrated viral DNA using primer sets specific for HIV-1 LTR and human Alu sequences with a FAM-TAMRA probe specific for the U5 region of the LTR. Experiments were initially

performed at an MOI of 1; however, after incubation of epithelial cells with R5 or X4 virus for 48 h, no integration was detected in either cell line (data not shown). Therefore, further experiments were carried out at increased MOI's, which also showed that both R5 (MOI of 7.5) and X4 (MOI of 10) virus failed to integrate into either TR146 or FaDu cells, whereas in the control cell lines (NP2-R5 and C8166) amplification of the integration product was detected after 28 and 32 cycles, respectively (Table 2). To confirm that lack of viral integration into the epithelial cell genome was not due to the presence of insufficient amounts of HIV-1, a final experiment using X4 virus at an MOI of 140 was performed, which also failed to produce detectable levels of HIV-1 integration (Table 2).

Productive HIV-1 infection is not restricted when HIV-1 enters via the endocytic pathway

Although we had convincing data that HIV-1 does not integrate and establish a productive infection in TR146 and FaDu cells, we sought to determine whether epithelial cells possessed the cellular machinery to support productive infection if conventional receptor-mediated entry mechanisms were by-passed. To test this, we utilized the three-plasmid expression system as described above [58] to generate VSV-G protein-pseudotyped HIV-1 vectors encoding GFP. By utilizing the endocytic entry of VSV-G, we found strong GFP fluorescence by flow cytometry in both TR146 (22%) and FaDu (38%) epithelial cells, at levels moderately lower than TZM-bl cells (50%) (Fig. 5). The difference in TR146 and FaDu infection efficiency may be due to the faster growth rate of FaDu cells over TR146 cells. Irrespective, the data indicate that epithelial cells are able to support productive HIV-1 infection if HIV-1 enters via the endocytic pathway. Addition of the HIV-1 reverse transcriptase inhibitor AZT abolished GFP fluorescence with the VSV-G pseudotyped virus, indicating the specificity of HIV-1 production in both epithelial cell types.

Transfer of captured HIV-1 from epithelial cells to permissive cells

One proposed mechanism of HIV-1 transmission across mucosal surfaces is via transfer of infections virus to underlying permissive cells post-capture by epithelial cells. Given that both R5 and X4 virus can be captured by oral epithelial cells (Fig. 3) but does not result in integration (Table 2) or productive viral infection (Fig. 4), we determined whether immobilized HIV-1 remained infectious post-capture. TR146 and FaDu cells were incubated with R5 and X4 virus for 24 h to allow for viral binding and following extensive washing, TZM-bl indicator cells were added for up to a further 48 h. Transfer of virus from TR146 and FaDu cells to TZM-bl cells would result in β -galactosidase activation and the appearance of blue foci. Fig. 6 indicates the presence of blue foci, most clearly at the border between epithelium and overlaid TZM-bl cells, which demonstrates that R5 and X4 virus can be transferred from the surface of both buccal and pharyngeal cells to permissive cells. These experiments were performed with multiple controls including absence of HIV-1 and incubation of TZM-bl cells with unconditioned and conditioned medium, but none of these resulted in blue foci, thus indicating that the blue foci were the result of R5 and X4 virus transfer.

Discussion

Although most HIV infections worldwide are transmitted through mucosal surfaces, their transmission through the oral mucosa and its secretions is thought to be uncommon [6]. Given that the oral cavity is a prime site for HIV-1 transmission in nursing infants and during oral-genital contact, it is profoundly important to understand the fate of HIV-1 after exposure to oral tissues. One view is that HIV-1 can directly infect epithelial cells with low efficiency, thereby establishing a primary infection. Another view is that HIV-1 is captured by epithelial cells and subsequently transcytosed or transferred to permissive cells in the sub-mucosa, thereby disseminating the virus in the body. Using buccal (TR146) and pharyngeal (FaDu) epithelial cells, our study supports the second view, as both cell types were able to capture and transfer infectious virus to permissive cells, which *in vivo* would permit infection of immune cells in the sub-mucosa and dissemination of HIV-

1 in the body. We also demonstrate that following HIV-1 binding, viral genome integration and other markers for potential *de novo* virus production, such as mRNA transcription and viral protein production do not occur in oral epithelial cells. However, epithelial cells possess the cellular machinery to support HIV-1 replication since the above-mentioned post-entry processes are not restricted if conventional HIV-1 entry is circumvented through endocytic pathways. Therefore, we propose that oral epithelial cells may play an important function in HIV-1 dissemination through their ability to bind HIV-1 and transfer viable virus to permissive cells and may support productive infection if receptor-mediated HIV-1 entry is circumvented.

HIV-1 binding requires expression of canonical (CD4, CCR5, CXCR4) or non-canonical (e.g. DC-SIGN, GalCer and HSPG's) receptors on the host cell surface. Using qualitative and quantitative analyses we demonstrate that both R5 and X4 virus are able to bind directly to TR146 and FaDu epithelial cells. However, binding appears to be independent of canonical HIV-1 receptor expression, since extremely low or undetectable levels of CD4, CCR5 and CXCR4 expression were found in both cell types, which is in concordance with most studies investigating expression of these receptors in epithelial cells [7,19,20,22,43,48]. Rather, viral binding is probably mediated via non-canonical receptors such as GalCer and HSPG's, given their greater expression levels in both buccal and pharyngeal epithelial cells, but unlikely to be via DC-SIGN which was expressed at low levels. Interestingly, FaDu cells expressed high levels of the HSPG syndecan-4, which was undetectable in TR146 cells. Given that X4 and R5 virus appear to bind FaDu cells more efficiently than TR146 cells, HIV-1 may preferentially or more efficiently bind the pharyngeal site of the oral cavity via syndecan-4. The utilization of both protein (e.g. HSPG) and non-protein (e.g. GalCer) moieties by HIV-1 to bind oral epithelial cells is supported by the fact that R5 and X4 binding was reduced but not abolished after trypsin digestion. Notably, X4 virus may preferentially utilize non-protein moieties since a greater number of X4 virus remained attached after trypsin digestion as compared with R5 virus. Also, while most primary HIV-1 infections occur with R5 virus [59] and some studies have found a preference for R5 selection in oral epithelial cells via cell-associated [48]

and cell-free systems [43], our extensive binding data indicate no such preference in buccal and pharyngeal epithelial cells as both R5 and X4 virus appear to bind equally well to either cell type. This study did not determine the efficiency of HIV-1 retention in comparison with other immune cells such as dendritic cells, since the purpose of these experiments was solely to determine whether HIV-1 binds epithelial cells.

Whether viral entry and genome integration occurs in oral epithelial cells is currently unclear, but this may be reflective of the experimental procedures used in different studies. One study using immortalized OKF6/TERT-2 cells (floor of mouth) showed that HIV-1 may directly integrate into oral epithelial cells without establishing a productive infection [21]. However, the lack of DNase treatment of virus stocks prior to infection or DpnI treatment of DNA samples after infection may not have been sufficient to remove potential plasmid contamination. Although nuclear extracts were used and heat-inactivated controls included, this may not have been sufficient to prevent false positives for viral integration. HIV-1 integration may also occur through utilization of the host cell recombination machinery, since mutations in HIV-1 integrase resulting in a defective enzyme do not necessarily eliminate provirus formation [60]. Other studies have demonstrated that integration and productive infection by X4 virus but not R5 virus occurs in primary gingival cells [18]. However, these cells were shown to express significant levels of CXCR4 and GalCer, which together can be used as alternative receptors to CD4. Using a sensitive real-time PCR assay we provide strong evidence that both R5 and X4 virus are unable to integrate their genomes into the DNA of both TR146 and FaDu epithelial cells. The samples were rigorously treated with DNase and DpnI digestion to prevent contaminating HIV-1 DNA; the method also measures the amount of carry-over DNA, as all samples are quantified relative to a matched heat-inactivated control.

To further study the fate of the virus after binding to oral epithelial cells we employed three different approaches: HIV-1 gp160 pseudotyped virus, detection of spliced HIV-1 *tat* mRNA, and *de novo* production of p55 gag protein. All three suggested that HIV-1 infection, *de novo* HIV-1 protein production and viral assembly are not supported in either epithelial cell type. However,

these assays do not ascertain if entry or internalization of the virus occurred since the *tat* mRNA PCR was not sensitive enough to detect the presence of low levels of spliced *tat* mRNA, which can be expressed from non-integrative viral DNA [61,62]. These observations, together with the general absence of CD4/CXCR4/CCR5 expression in both cell types, support the view that productive HIV-1 infection requires canonical receptor expression on the host cell. Despite this, our findings are in contrast with some studies demonstrating productive viral infection in epithelial cell lineages isolated from tonsillar tissue [44-46], adenoids [43], salivary glands [47,48] and primary gingival keratinocytes [18,43]. However, the findings of these studies appear to correlate with greater expression of CXCR4 and/or GalCer in epithelial cells from these oral sites. Furthermore, the *in vivo* relevance of one study demonstrating productive infection of X4 virus but not R5 virus in primary gingival cells [18] was questioned by Quinones-Mateu [20], as it used the artificial compound polybrene to promote HIV-1 viral entry into the epithelial cell [18] (see below).

Several studies have reported that HIV-1 is endocytosed by epithelial cells [63], which can then lead to productive infection. To address this we utilized a GFP-encoding VSV-G pseudotyped HIV-1 virus, which utilizes the endocytic pathway for cell entry and by-passes conventional CD4 receptor-mediated entry mechanisms. This virus was able to establish a productive infection in both TR146 and FaDu cells that could be inhibited with AZT, demonstrating two key points. First, HIV-1 does not enter epithelial cells via the endocytic pathway (otherwise a productive infection would ensue with live virus) and that HIV-1 binding is probably mediated through non-canonical viral-specific receptors. Second, epithelial cells possess the cellular machinery to assemble and secrete infectious viral progeny if receptor-mediated entry is by-passed (as VSV-G pseudotyped HIV-1 virus establishes productive infection). Together with the fact that HIV-1 infection of TZM-bl cells (epithelial-like cells expressing CD4, CXCR4 and CCR5) also results in the assembly and secretion of infectious viral progeny, our data suggests that oral epithelial cells are able to support productive viral infection if HIV-1 is able to gain entry into the cell. This may explain why the use of polybrene led to productive HIV-1 infection in primary gingival epithelial cells [18] (see above).

Our findings raise the intriguing possibility that if conditions arise *in vivo* that enable receptor-mediated entry to be by-passed, for example during inflammatory responses, productive HIV-1 infection may be supported in oral epithelial cells.

Although under normal conditions productive HIV-1 infection does not appear to occur in epithelial cells, the virus remains attached to epithelial cells giving rise to the possibility that captured virus may be transmitted to permissive cells in the underlying mucosa. Utilizing a cell overlay experiment we show that HIV-1 retains infectivity on the epithelial cell surface and can be readily transferred to TZM-bl cells to establish a productive infection. Transfer of virus to permissive cells has also been observed for other oral epithelial cell lineages [18,21,36,52] and appears to occur in a CD4/CCR5/CXCR4 and GalCer independent manner. These findings have major implications for HIV-1 infection *in vivo* as this may provide a window of opportunity for the virus to be transferred to intraepithelial T lymphocytes, dendritic cells and tissue macrophages in the sub-mucosa, thereby establishing an acute infection and disseminating the virus in the body. Indeed, evidence that *trans* infection might occur *in vivo* has been provided in simian experiments, which demonstrated systemic viral dissemination 24 h after atraumatic exposure of the oral mucosa to SIV-1 [16]. Furthermore, using *ex vivo* tissue explants Tugizov *et al.* [64] recently demonstrated that HIV-1 is efficiently transcytosed through adult and fetal polarized oral epithelial cells, although only virions emerging after transcytosis from fetal epithelial cells appeared to be infectious.

In summary, we demonstrate that HIV-1 is able to bind to oral epithelial cells via non-canonical viral-specific receptors but that genome integration and productive viral infection does not occur. However, oral epithelial cells possess the cellular machinery to support productive HIV-1 infection if the virus enters via the endocytic pathway or if conventional entry mechanisms are by-passed. Once captured, HIV-1 also remains infectious on the surface of epithelial cells, which may facilitate direct viral transfer to permissive cells in the sub-mucosa thereby establishing acute infection. Therefore, although often considered a rare site of HIV-1 infection, the oral mucosa may have been overlooked as a potential route of viral transmission and mediator of systemic infection.

Of vital importance for future work is the identification of the non-canonical viral-specific receptors that allow HIV-1 binding. Manipulation of these receptors may have the potential to prevent epithelial transfer of HIV-1 and prevent productive infection in permissive cells.

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Figure Legends

Figure 1. Basal HIV-1 receptor mRNA expression in resting oral epithelial cells. TR146, FaDu and TZM-bl cells were examined for mRNA expression of CD4, CCR5, CXCR4, DC-SIGN and the HSPG's syndecan-1 and -4 by quantitative RT-PCR. Data are presented as mRNA transcripts (arbitrary units) normalized to β -actin in a minimum of three independent experiments. Bars indicate \pm standard deviation from the mean.

Figure 2. Basal HIV-1 receptor surface expression in resting oral epithelial cells. TR146, FaDu, TZM-bl and NP2-R5 and -X4 expressing cells were examined for surface expression of CD4, CCR5, CXCR4, DC-SIGN, GalCer and HSPG's by flow cytometry using monoclonal primary antibodies specific to each receptor with a FITC-labeled secondary antibody. HSPG's were not analyzed in NP2-R5 or -X4 expressing cells. Data are presented as percentage of cells expressing each receptor in a minimum of three independent experiments. Bars indicate \pm standard error of the mean.

Figure 3. HIV-1 R5 and X4 binding to oral epithelial cells. Primary epithelial cells, TR146, FaDu and TZM-bl cells were incubated with cell free YU2 (R5) or LAI (X4) infectious virus overnight under identical conditions and extensively washed. (A) Detection of p24 gag protein by Western blotting using α -actin as a loading control; (B) Detection of immobilized virus on the cell surface by flow cytometry using a Cy5-labeled anti-human secondary antibody to detect HIV-1 gp120 monoclonal primary detected on APC channel. Gates set around unlabelled cell control, this number is then set to 0 and the percent cells shifted to the right of the gate are deemed positive. Then, virally exposed, labelled cell percentages are subtracted from control (not virally exposed) labelled

cell percentages to obtain the % fluorescence values shown. Data are representative of four independent experiments and bars indicate \pm standard deviation from the mean (C) Detection of packaged HIV R5 RNA by amplification of the HIV-1 *pol* gene using nested PCR. (D) % reduction in detection of immobilized virus on the cell surface by flow cytometry after trypsin treatment. Virally exposed cells are compared with cells labelled with secondary alone. Data sets are representative of three independent experiments.

Figure 4. Post-integration HIV-1 mRNA transcription and *de novo* viral protein production in oral epithelial cells. (A) Detection of spliced HIV-1 *tat* mRNA in TR146, FaDu and TZM-bl control cells by PCR 24 h post-infection with YU2 (R5) or LAI (X4) infectious virus. (B) p55 gag protein detection in TR146, FaDu and TZM-bl control cells by Western blot after 24 h infection with R5 (YU2) and LAI (X4) virus. (C) Infection of TR146, FaDu and NP2-R5/X4 control cells with GFP-linked single-cycle X4, R5 and dual tropic HIV-1 gp160 pseudotyped virus and detection of GFP incorporation into epithelial cell DNA by flow cytometry. Data are representative of three independent experiments.

Figure 5. HIV-1 entry via the endocytic pathway results in productive viral infection in oral epithelial cells. Two fold serial dilutions of VSV-G pseudotyped HIV-1 (MOI 1 – 0.125) were added to TR146, FaDu and TZM-bl control cells. Infection is measured by flow cytometry as % GFP expression (black bars). The effect of AZT (500mM) on GFP expression was also measured at the highest virus inoculum (white bar). Data are representative of three independent experiments

Figure 6. Transfer of captured HIV-1 from epithelial cells to permissive cells. TR146 and FaDu and TZM-bl cells were incubated with R5 (YU2) and LAI (X4) virus for 24 h and following extensive washing TZM-bl indicator cells were added for a further 48 h. Controls included FaDu

and TR146 cells without the addition of virus. Data are representative of three independent experiments.

Table 1. Primer sets detecting gene expression of HIV-1 associated receptors

Gene	Primer	Sequence	Annealing temp. (°C)	Extension temp. (°C)	Product (bp)
CD4	Forward	5'- ACTAAAGGTCCATCCAAGCTGA —3'	60	75	151
	Reverse	5'- GCAGTCAATCCGAACACTAGCA —3'			
CCR5	Forward	5'- TGGACCAAGCTATGCAGGTG —3'	58	75	240
	Reverse	5'- CGTGTCACAAGCCCACAGAT —3'			
CXCR4	Forward	5'- CCTCATCCTGGCTTTCTTCG —3'	60	75	285
	Reverse	5'- GAATGTCCACCTCGCTTTCC —3'			
DC-SIGN	Forward	5'- TCAAGCAGTATTGGAACAGAGGA —3'	60	75	136
	Reverse	5'- CAGGAGGCTGCGGACTTTTT —3'			
Syndecan-1	Forward	5'- TGAAACCTCGGGGAGAATAC —3'	60	75	171
	Reverse	5'- GGTACAGCATGAAACCCACC —3'			
Syndecan-4	Forward	5'- CAGGGTCTGGGAGCCAAGT —3'	58	72	129
	Reverse	5'- GCACAGTGCTGGACATTGACA —3'			
β-actin	Forward	5'- CATGTACGTTGCTATCCAGGC —3'	58	75	250
	Reverse	5'- CTCCTTAATGTCACGCACGAT —3'			

Table 2. Detection of integrated HIV-1 genome in oral epithelial cells

Cell line	R5-YU2 (MOI = 7.5)	X4-LAI (MOI = 10)	X4-LAI (MOI = 140)
FaDu	ND	Not included	ND
TR146	ND	ND	ND
C8166 ^a	n/a	+ (Ct = 32.7)	+ (Ct = 33.5)
NP2-R5 ^b	+ (Ct = 28.8)	n/a	n/a

^a C8166 cells express CXCR4 and were used for X4 viral infections only.

^b NP2-R5 cells express CCR5 and were used for R5 viral infections only.

+, Integrated HIV-1 product detected (cycle threshold detection in brackets).

ND, Integrated HIV-1 product not detected.

Figure 1
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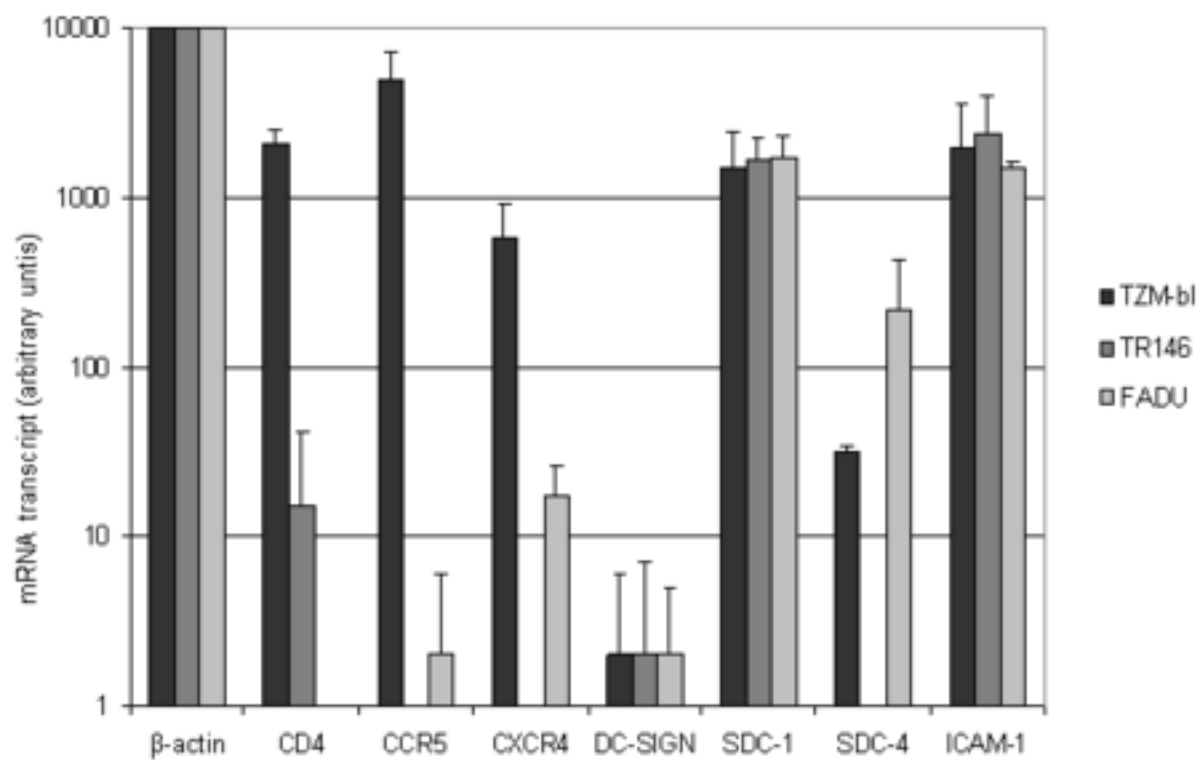


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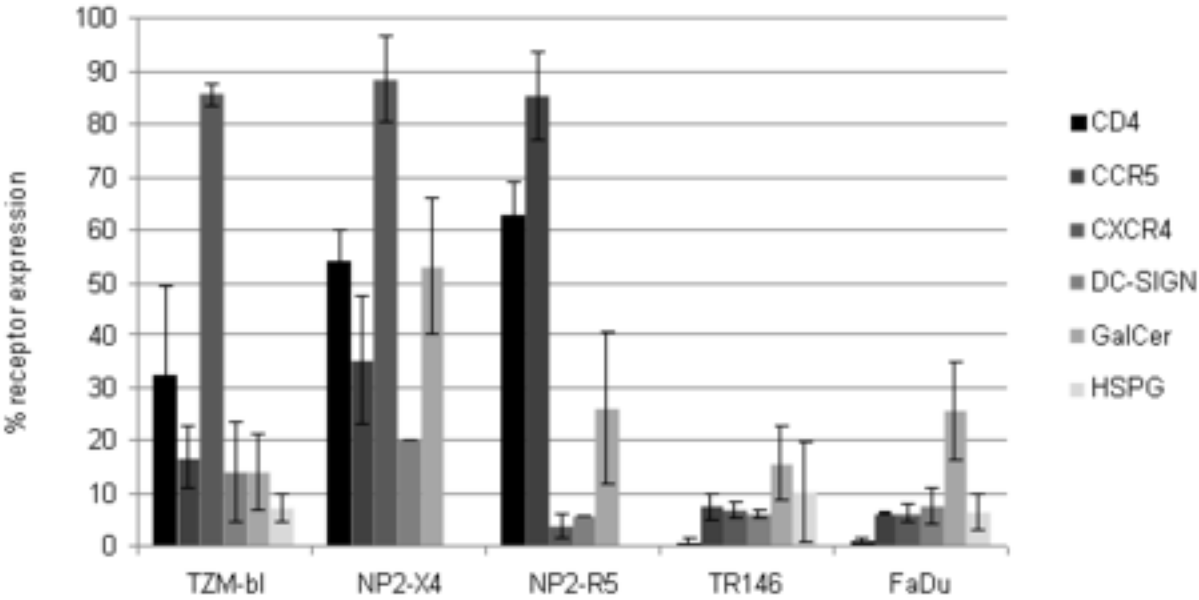


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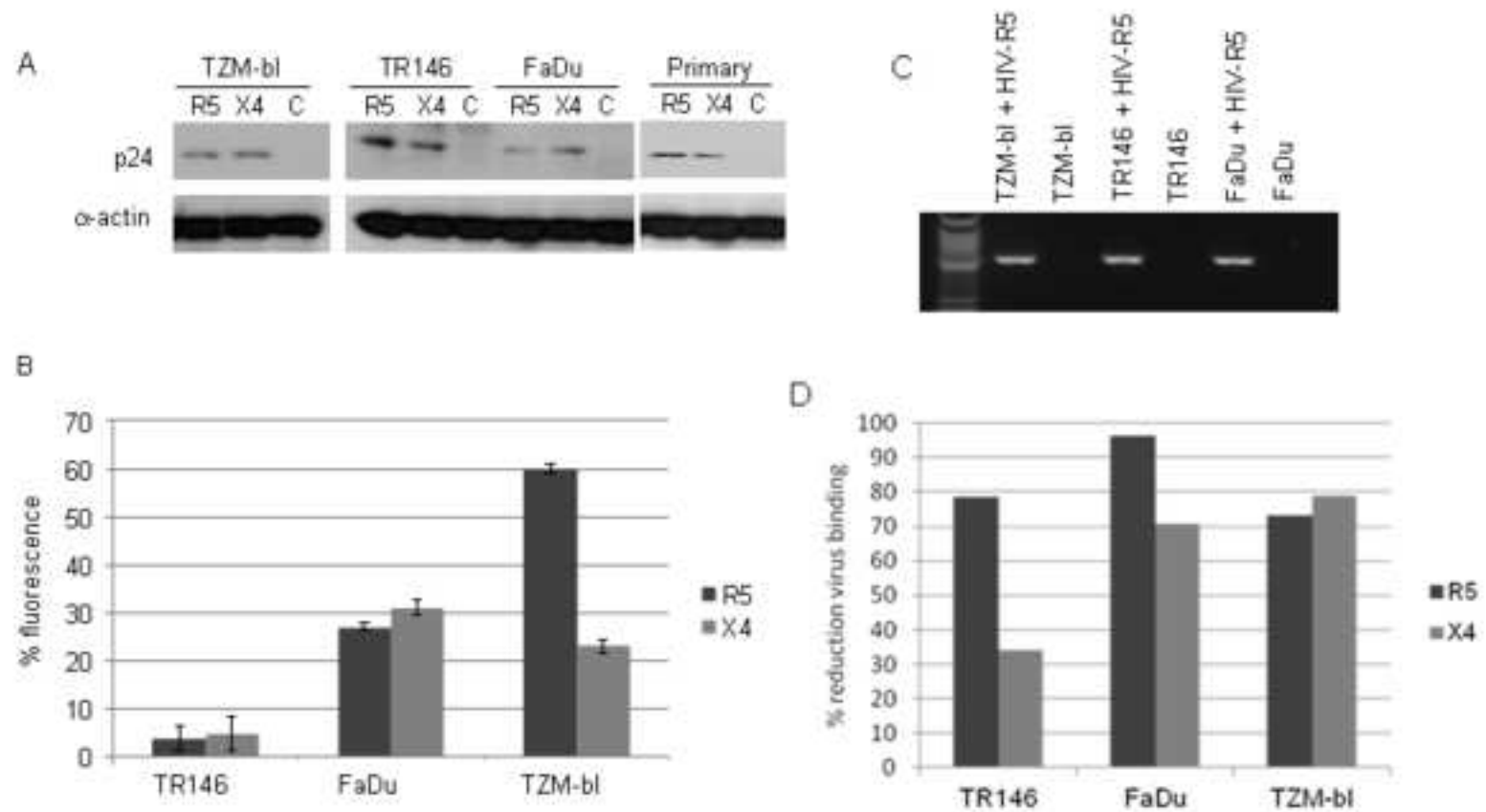


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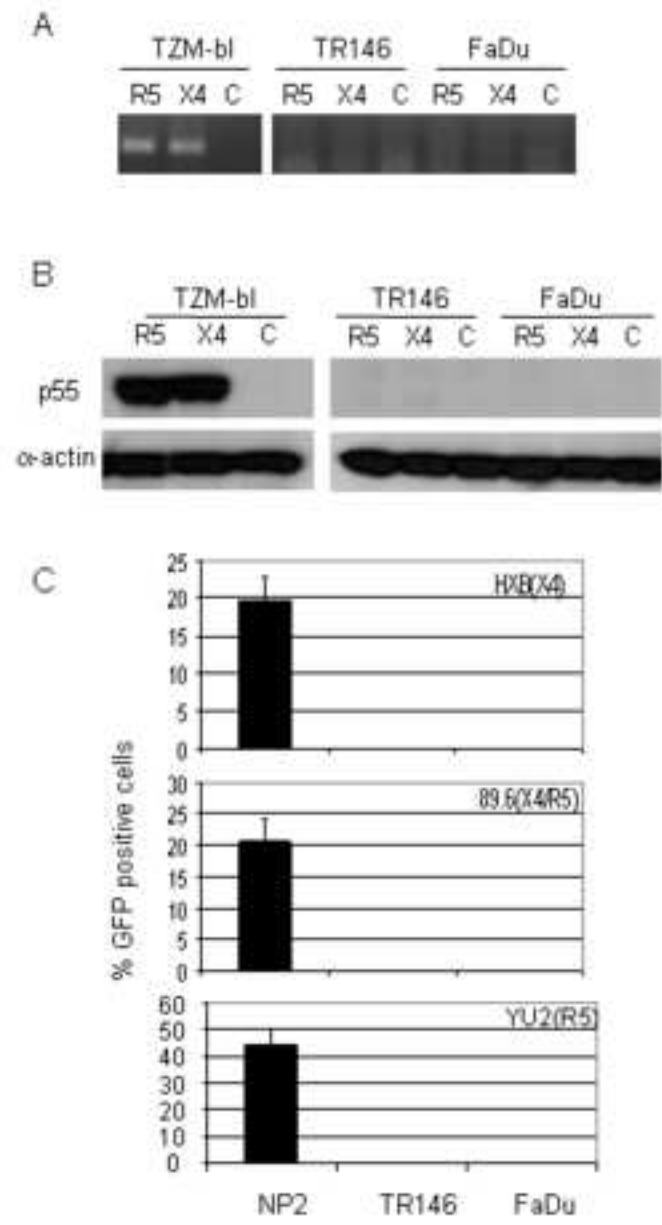


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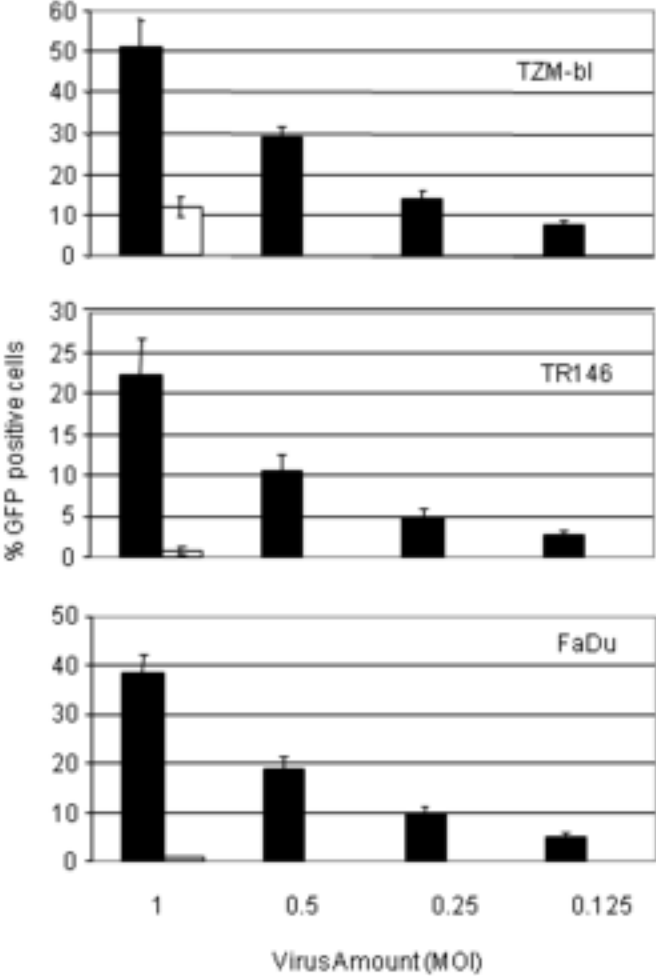
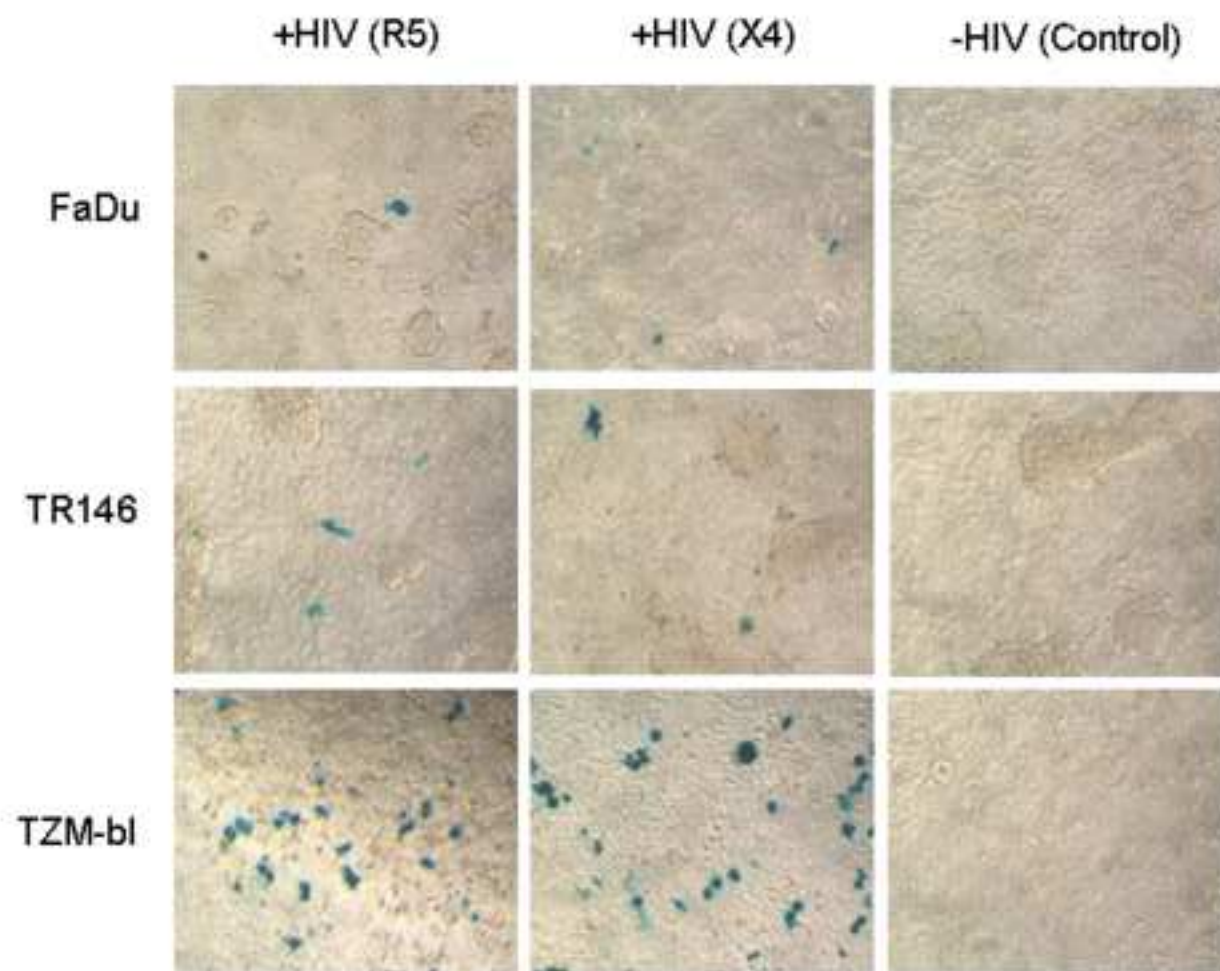


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Abstract: The vast majority of HIV-1 transmission occurs across the vaginal mucosa. In this study we utilized a vaginal epithelial cell line (A431) to determine HIV-1 receptor expression, HIV-1 capture and transfer to permissive cells, and viral integration and productive infection. Using flow cytometry and Western blotting to detect gp120 and p24, we demonstrate that whilst A431 cells do not express discernable levels of CD4, CCR5 or CXCR4, they do capture both X4 and R5 virus probably via non-canonical receptors. Once captured, HIV-1 remains infectious and can be transferred to permissive TZM-bl reporter cells. However, HIV-1 integration and productive infection, as measured by real-time PCR, presence of early gene mRNA transcripts, and de novo protein production was not detected. Notably, VSV-G-packaged HIV-1 was replication competent in A431 cells. Thus, while productive HIV-1 infection is unlikely to occur in vaginal cells, they may provide a potential route to establish systemic infection.

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To Virology,

Dear Editor,

Please consider the enclosed manuscript entitled "Capture and transfer of infectious HIV-1 by vaginal epithelial cells" for publication in Virology.

The majority of HIV-1 infections worldwide are acquired via mucosal surfaces, predominantly across the female genital tract. However, there is ambiguous literature on whether HIV-1 binds, enters and integrates into vaginal epithelial cells, whether productive infection ensues, and whether sequestered infectious virus can be transferred to permissive immune cells. We utilized an epithelial cell line of stratified squamous cell origin (A431), which is representative of a site (vulvovaginal) that is rarely investigated with regard to vaginal transmission but is one of the first cell types that is likely to come into contact with HIV-1.

Our extensive data demonstrate that HIV-1 X4 and R5 virus can readily bind vaginal epithelial cells (probably via non-canonical viral-specific receptors) but that integration of the viral genome into epithelial cell DNA and *de novo* virus production does not ensue. Thus, productive infection does not occur in vaginal epithelial cells. However, notably, vaginal epithelial cells possess the cellular machinery to support productive HIV-1 infection if the virus enters via the endocytic pathway or if conventional entry mechanisms are by-passed. Also, once captured, HIV-1 remains infectious on the surface of vaginal epithelial cells and can be transferred to permissive cells, which has important implications *in vivo*. Thus, the vaginal route may thus facilitate direct HIV-1 transfer to permissive cells in the sub-mucosa, thereby establishing acute infection by disseminating HIV-1 in the body.

We would like to stress the novelty aspects of our work. First, the A431 epithelial cell line has not previously been used to investigate HIV-1 binding or productive infection; thus, the entire data set is novel. Second, to our knowledge gp120 detection on the surface of epithelial cells and trypsin resistance to gp120 detection as measured by flow cytometry is also novel. Third, the viral integration experiments using the qPCR based primer-probe method has not previously been used in vaginal epithelial studies. Finally, we are unaware of any study using the VSV-G system to demonstrate that vaginal epithelial cells possess the cellular machinery to support productive HIV-1 infection if the virus enters via the endocytic pathway.

Sincerely,

Dr Julian Naglik

Capture and transfer of infectious HIV-1 by vaginal epithelial cells

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Running title: HIV-1 capture and transfer by vaginal epithelial cells

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1 **Abstract**

2 The vast majority of HIV-1 transmission occurs across the vaginal mucosa. In this study we utilized
3 a vaginal epithelial cell line (A431) to determine HIV-1 receptor expression, HIV-1 capture and
4 transfer to permissive cells, and viral integration and productive infection. Using flow cytometry
5 and Western blotting to detect gp120 and p24, we demonstrate that whilst A431 cells do not express
6 discernable levels of CD4, CCR5 or CXCR4, they do capture both X4 and R5 virus probably via
7 non-canonical receptors. Once captured, HIV-1 remains infectious and can be transferred to
8 permissive TZM-bl reporter cells. However, HIV-1 integration and productive infection, as
9 measured by real-time PCR, presence of early gene mRNA transcripts, and *de novo* protein
10 production was not detected. Notably, VSV-G-packaged HIV-1 was replication competent in A431
11 cells. Thus, while productive HIV-1 infection is unlikely to occur in vaginal cells, they may provide
12 a potential route to establish systemic infection.

13
14 **Keywords**

15 HIV-1, epithelium, mucosal, vaginal, expression, receptor, integration, infection, gp120, p24

16

1 **Introduction**

2 Heterosexual transmission accounts for the majority of new HIV-1 infections (Hladik & McElrath,
3 2008). Both men and women have been shown to have detectable HIV-1 in seminal fluid and
4 cervicovaginal secretions (Dulioust et al., 1998; Coombs et al., 1998; Goulston et al., 1998;
5 Goulston et al., 1996). Studies have shown that cell-free and cell-associated HIV-1 can establish
6 mucosal infection (Phillips et al., 1998; van Herrewege Y et al., 2007), and macaque and human
7 studies indicate that transmission is facilitated by the presence of HIV-1 target cells (dendritic cells,
8 Langerhans cells, CD4⁺ T cells and macrophages) in the ectocervix and vagina as well as in the
9 endocervix and uterus (Yeaman et al., 1998; Shen et al., 2011; Ballweber et al., 2011; Kaldensjo et
10 al., 2011; Shen et al., 2009; Miller & Shattock, 2003; Khanna et al., 2002; Bhoopat et al., 2001; Hu
11 et al., 2000; Hu et al., 1998; Spira et al., 1996; Maher et al., 2005; Hu et al., 2004; Greenhead et al.,
12 2000; Hladik et al., 2007; Zhang et al., 1999). HIV-1 entry into permissive cells requires the
13 expression of the CD4 receptor and a chemokine fusion co-receptor (CCR5 for R5-tropic or
14 CXCR4 for X4-tropic) (Pierson & Doms, 2003). However, most studies indicate that there is a
15 general absence or low level surface expression of CD4, CCR5 and CXCR4 in vaginal epithelial
16 cells (Berlier et al., 2005; Bobardt et al., 2007; Dezzutti et al., 2001), although data for CXCR4
17 surface expression is somewhat varied (Berlier et al., 2005; Yeaman et al., 2004).

18 Despite these receptor dependencies, HIV-1 may also infect CD4⁻ cells such as
19 oligodendrocytes (Harouse et al., 1991), fibroblasts (Tateno et al., 1989) and spermatozoa (Baccetti
20 et al., 1994), as well as epithelial cells from the gastrointestinal tract (Asmuth et al., 1994) and
21 thymus (Braun et al., 1996). Thus, HIV-1 may utilize several non-canonical receptor mechanisms
22 for binding and entry into host cells. The viral envelope protein gp120 has been shown to bind
23 several cell-surface molecules including DC-SIGN (dendritic cell-specific intercellular adhesion
24 molecule-3-grabbing non-integrin) (Geijtenbeek et al., 2000; van Kooyk & Geijtenbeek, 2003),
25 heparan sulphate proteoglycans (HSPGs) such as syndecan-1 (Bobardt et al., 2003; Wu et al., 2003;
26 Mondor et al., 1998) and GalCer (glycosphingolipid galactosylceramide) (Alfsen & Bomsel, 2002;

1 Delezay et al., 1997; Harouse et al., 1991). HSPGs and GalCer are commonly expressed on
2 epithelial cells and may promote HIV-1 binding and transport across the vaginal epithelium
3 (Bobardt et al., 2007; Wu et al., 2003; Weinberg et al., 2011; Dezzutti et al., 2001; Yeaman et al.,
4 2004). Notably, mucosal transmission of HIV-1 most frequently occurs with R5-utilizing viruses
5 (Margolis & Shattock, 2006; Zhu et al., 1993); however, a full and satisfactory explanation for R5
6 selectivity has not yet been provided.

7 One mechanism of HIV-1 transmission across the vaginal mucosa is thought to occur through
8 sequestration of the virus by epithelial cells, followed by transfer to permissive cells to establish a
9 primary infection (Yeaman et al., 1998; Shen et al., 2011; Ballweber et al., 2011; Kaldensjo et al.,
10 2011; Shen et al., 2009; Miller & Shattock, 2003; Khanna et al., 2002; Bhooapat et al., 2001; Hu et
11 al., 2000; Hu et al., 1998; Spira et al., 1996; Dezzutti et al., 2001). Similarly, HIV-1 binding to
12 epithelial cells may directly impair barrier integrity facilitating entry (Fanibunda et al., 2011; Nazli
13 et al., 2010). However, the fundamental issue of whether vaginal epithelial cells can be productively
14 infected with HIV-1 remains controversial. Whilst some studies support the view that HIV-1 can
15 integrate into the vaginal epithelial genome and produce progeny virus (Phillips et al., 1998; Furuta
16 et al., 1994; Phillips et al., 1994; Tan et al., 1993), others discount this view (Dezzutti et al., 2001;
17 Greenhead et al., 2000; Wu et al., 2003; Spira et al., 1996). It has been proposed that HIV-1 may
18 also be sequestered in cytosolic endocytic compartments or transmitted by transcytosis (Hladik &
19 McElrath, 2008; Hocini & Bomsel, 1999; Hocini et al., 2001; Bobardt et al., 2007; Bomsel, 1997)
20 without establishing productive infection within epithelial cells. In addition, proviral DNA from X4
21 tropic viruses has been detected in vaginal epithelial cells (Berlier et al., 2005; Iversen et al., 1998),
22 suggesting that HIV-1 may potentially integrate into the epithelial genome even if this does not give
23 rise to productive infection.

24 Given the current ambiguous literature on this fundamentally important topic, we aimed to
25 determine whether HIV-1 binds, enters and integrates into vaginal epithelial cells, whether
26 productive infection ensues, and whether sequestered infectious virus can be transferred to

permissive cells. We utilized the A431 epidermoid carcinoma epithelial cell line of vulval (squamous) origin, which represents a site commonly exposed to HIV-1 during heterosexual intercourse but is rarely used to study HIV-1 life cycle events. We demonstrate that A431 cells were able to capture X4 and R5 virus but that integration of the viral genome into epithelial cell DNA and *de novo* virus production does not occur. Notably, VSV-G-packaged HIV-1 was replication competent in A431 cells. Finally, A431 cells were able to transfer infectious X4 and R5 virus to permissive TZM-bl reporter cells, which *in vivo* would represent transfer of virus to immune cells in the sub-mucosa and dissemination of HIV-1 in the body.

Results

HIV-1 receptor expression in vaginal epithelial cells

Surface receptor binding is the initial step of HIV-1 infection. Therefore, we first analyzed the gene expression levels of CD4, CCR5, CXCR4, DC-SIGN and the HSPG's syndecan-1 and syndecan-4 in A431 cells by quantitative PCR (Fig. 1). GalCer was excluded as this non-protein moiety can not be investigated by gene expression. This demonstrated the absence of CCR5 and CXCR4 expression in A431 cells but high expression in the control TZM-bl cells. Notably CD4 mRNA was detected, albeit approximately 12-fold lower than TZM-bl cells. In both cell types, DC-SIGN was minimally expressed but HSPG syndecan-1 was highly expressed. Syndecan-4 was expressed at intermediate levels, with approximately 5-fold greater expression in A431 cells as compared with TZM-bl cells.

Given that mRNA expression often does not correlate with protein expression, we next determined the surface expression of CD4, CCR5, CXCR4, DC-SIGN, GalCer, and HSPG's on oral epithelial cells by flow cytometry (Fig. 2). A431 cells expressed undetectable levels of CD4 and very low levels of CCR5 and CXCR4 (~6-8%), unlike the control TZM-bl cells which are HeLa cell derivatives engineered to express high levels of CD4 (40%), CCR5 (85%) and CXCR4 (85%). The A431 data suggests that the CD4 mRNA detected by gene expression (Fig. 1) did not translate

into surface CD4 protein expression. With regard to non-canonical receptors, both A431 and TZM-bl cells expressed low amounts of DC-SIGN (~5% and 16%, respectively). However, unlike TZM-bl cells, A431 cells expressed greater surface levels of GalCer (~75%) and HSPG's (35%).

Binding of HIV-1 to vaginal epithelial cells

Next, HIV-1 binding to vaginal epithelial cells was assessed. A431 and TZM-bl cells were incubated overnight with cell free YU2 (R5) or LAI (X4) infectious virus. After extensive washing the presence of captured virus was determined using three approaches. First, total protein was isolated and the presence of HIV-1 p24 gag protein determined by immunoblot analysis. p24 was present in A431 protein lysates at levels similar to that found with TZM-bl cells, indicating that both R5 and X4 virus are captured by vaginal epithelial cells (Fig. 3A). Second, we used a more quantitative approach by detecting attached virus by flow cytometry using a Cy5-labeled secondary antibody to detect a human monoclonal primary (F425 B4e8) that bound to HIV-1 gp120. Both R5 and X4 virus was detected on A431 cells (~10%), albeit at lower levels than TZM-bl cells (60-70%), demonstrating direct binding of infectious virus to vaginal epithelial cells (Fig. 3B). A second monoclonal primary F425 A1g8 antibody showed similar binding to the epithelial lines as F425 B4e8 (data not shown). Third, we assessed for the presence of packaged HIV RNA by amplification of the HIV-1 *pol* gene using nested PCR. This approach was used to confirm the p24 protein and whole virus binding data; therefore, we performed this experiment using only the R5 virus. Amplification of the HIV-1 *pol* gene indicated the presence of R5 virus on A431 cells in addition to TZM-bl cells (Fig. 3C). The Western blot (Fig. 3A) and PCR (Fig. 3C) data are qualitative and may not reflect differences in the efficiency of R5 and X4 HIV-1 binding to A431 cells, which was more apparent using the quantitative flow cytometry approach (Fig. 3B).

Finally, we determined whether binding of R5 and X4 virus to A431 and TZM-bl cells was trypsin sensitive after 5 min incubation with trypsin at 37°C by detecting gp120 using flow cytometry. Interestingly, while binding of both R5 and X4 virus to TZM-bl cells and R5 virus to

A431 was trypsin sensitive (~70-80% reduction in binding), X4 binding was more resistant, as binding was reduced by only ~15 % (Fig. 3D). This suggests that R5 virus interacts predominantly with trypsin-sensitive protein moieties on vaginal epithelial cells to mediate binding, whilst X4 virus predominantly utilizes non-protein moieties or trypsin-insensitive protein moieties. Taken together, the data demonstrate that both R5 and X4 virus directly bind vaginal epithelial cells, although differences may exist in the surface moieties used for attachment.

HIV-1 mRNA transcription and *de novo* viral protein production

Since HIV-1 was captured by A431 cells, we hypothesized that vaginal epithelial cells may support productive infection. This would constitute initiation of HIV-1 mRNA transcription, *de novo* viral protein production and, ultimately, assembly of new viral progeny. To investigate this we utilized four different approaches. First, we used a PCR-based system to detect spliced HIV-1 *tat* mRNA in A431 cells 24 h post-infection with R5 and X4 virus. The presence of high levels of spliced *tat* mRNA would indicate HIV-1 integration and *de novo* production of viral mRNA transcripts, which in permissive cells is representative of a productive HIV-1 infection resulting in production of new viral progeny. However, we found that spliced *tat* mRNA was only detected in control TZM-bl cells and not in A431 cells (Fig. 4A). Second, we assessed protein lysates of A431 and TZM-bl cells for p55 gag protein by Western blot 24 h post-infection with YU2 (R5) or LAI (X4) virus. Detection of p55 gag protein would only be observed if *de novo* viral protein production had occurred. Fig. 4B shows p55 gag protein expression only in TZM-bl cells but not A431 vaginal epithelial cells. Third, since most assays were undertaken 24 – 48 h post-infection with HIV-1, we also assessed for HIV-1 progeny after day 4 and 7 in case a longer time period was required to establish a productive infection in vaginal epithelial cells. However, neither R5 nor X4 culture supernatants at either time point induced blue foci formation (demonstrating HIV-LTR driven β -galactosidase production), indicating that no new infectious virions were produced (data not shown). Fourth, we utilized a three-plasmid expression system (Naldini et al., 1996) to generate HIV-based vectors pseudotyped

with HIV-1 envelopes, YU2 (R5), HXB (X4) or 89.6 (dual tropic). Supernatants containing these replication defective retroviral particles were used to transduce A431 and NP2-R5/X4 cells. NP2 cells were used as the positive control because receptor expression is maintained using selective media and thus efficiency of viral infection was greater than with TZM-bl cells. Unlike the NP2-X4/R5 cells, A431 cells failed to drive expression of the GFP reporter gene, resulting in undetectable GFP fluorescence up to 48 h post-infection (Fig. 4C). Taken together, these extensive data sets indicate that productive HIV-1 infection does not occur in A431 vaginal epithelial cells.

Integration of HIV-1 into the vaginal epithelial genome

Although R5 and X4 virus was unable to productively infect A431 cells (Fig. 4), it was possible that, post-capture (Fig. 3), HIV-1 was able to gain entry and integrate into the epithelial genome to establish a latent infection. To test this we performed a real-time PCR assay to detect integrated viral DNA using primer sets specific for HIV-1 LTR and human Alu sequences with a FAM-TAMRA probe specific for the U5 region of the LTR (Mbisa et al., 2009). Initial experiments performed at an MOI of 1 indicated no integration by either R5 or X4 virus after 48 h (data not shown). Further experiments demonstrated that increasing the MOI to 7.5 (X4) and 10 (R5) also failed to permit viral integration into A431 cells, whereas in the control cell lines (NP2-R5 and C8166) amplification of the integration product was detected after 29 and 33 cycles, respectively (Table 2). To confirm that lack of viral integration into A431 cells was not due to the presence of insufficient amounts of HIV-1, a final experiment using X4 virus at an MOI of 140 was performed, which also failed to produce any detectable levels of HIV-1 integration (Table 2). These data demonstrate that HIV-1 X4 and R5 do not integrate into the vaginal epithelial genome.

Productive infection occurs in A431 cells when HIV-1 enters via the endocytic pathway

Although R5 and X4 virus does not integrate and establish a productive infection in A431 cells, we sought to determine whether vaginal epithelial cells possessed the cellular machinery to support

productive HIV-1 infection if receptor-mediated entry mechanisms were by-passed. Therefore, we utilized the same three-plasmid expression system as described above (Naldini et al., 1996) to generate VSV-G protein-pseudotyped HIV-1 vectors encoding GFP. By utilizing the endocytic entry of VSV-G, strong GFP fluorescence was observed by flow cytometry in A431 cells (32%) at levels only slightly lower than TZM-bl cells (50%) (Fig. 5). These data indicate that vaginal epithelial cells are able to support productive HIV-1 infection if the virus enters via the endocytic pathway. Addition of the HIV-1 reverse transcriptase inhibitor AZT abolished GFP fluorescence with the VSV-G pseudotyped virus in both A431 and TZM-bl cells, indicating the specificity of *de novo* HIV-1 production.

HIV-1 transfer from vaginal epithelial cells to permissive TZM-bl cells

A common route of HIV-1 transmission across the vaginal mucosa is thought to be via transfer of infectious virus to underlying permissive cells post-capture by epithelial cells. As R5 and X4 virus are captured by vaginal epithelial cells (Fig. 3) but do not integrate (Table 2) or establish productive viral infection (Fig. 4), we next determined whether immobilized HIV-1 remained infectious post-capture. A431 cells were incubated with R5 and X4 virus for 24 h to allow for viral binding and following extensive washing, TZM-bl indicator cells were added for up to a further 48 h. Transfer of virus from A431 cells to TZM-bl cells and their subsequent infection would result in β -galactosidase activation and the appearance of blue foci. Fig. 6 indicates the presence of blue foci (arrows shown for A431/R5), which demonstrates that R5 and X4 virus can be transferred from the surface of vaginal cells to permissive cells. Experiments were performed with additional controls including absence of HIV-1 and incubation of TZM-bl cells with conditioned medium, none of which resulted in the appearance of blue foci.

Discussion

1 The majority of HIV-1 infections worldwide are acquired via mucosal surfaces, predominantly
2 across the female genital tract (Hladik & McElrath, 2008). As such, it is profoundly important to
3 understand the fate of HIV-1 after exposure to vaginal mucosa. One view is that vaginal HIV-1
4 transmission occurs either by direct infection of permissive cells within the vaginal mucosa or
5 sequestration of the virus by epithelial cells followed by transfer to permissive cells (Yeaman et al.,
6 1998; Shen et al., 2011; Ballweber et al., 2011; Kaldensjo et al., 2011; Shen et al., 2009; Miller &
7 Shattock, 2003; Khanna et al., 2002; Bhoopat et al., 2001; Hu et al., 2000; Hu et al., 1998; Spira et
8 al., 1996; Dezzutti et al., 2001; Hladik & McElrath, 2008; Bobardt et al., 2007; Hocini & Bomsel,
9 1999; Hocini et al., 2001; Bomsel, 1997). However, another view is that vaginal epithelial cells may
10 be productively infected with HIV-1 or that the virus establishes a latent (non-productive) infection
11 by integrating into epithelial genome (Phillips et al., 1998; Furuta et al., 1994; Phillips et al., 1994;
12 Tan et al., 1993; Iversen et al., 1998; Berlier et al., 2005). Using vulvovaginal (A431) epithelial
13 cells, our study supports the first view, as epithelial cells were able to capture and transfer infectious
14 virus to permissive TZM-bl cells but are not productively infected, with no evidence of viral
15 genome integration. However, our data would also suggest that vaginal epithelial would be
16 productively infected by HIV-1 if conventional entry is circumvented, such as through endocytic
17 pathways. Therefore, we propose that vaginal epithelial cells role in HIV-1 dissemination is through
18 their ability to bind HIV-1 and transfer viable virus to permissive cells, which *in vivo* would permit
19 infection of immune cells in the sub-mucosa and establishment of a primary HIV-1 infection.

20 Using both qualitative and quantitative analyses we demonstrate that R5 and X4 virus is able
21 to bind directly to A431 vaginal epithelial cells. Binding appears to be independent of canonical
22 HIV-1 receptor expression, since extremely low or undetectable levels of CD4, CCR5 and CXCR4
23 expression were found in A431 cells, which is in concordance with other human studies (Berlier et
24 al., 2005; Dezzutti et al., 2001; Bobardt et al., 2007). This supports studies with primary vaginal
25 epithelial cells using inhibitors and neutralising antibodies, which indicate that HIV-1 binding is
26 independent of CD4, CXCR4 and CCR5 (Bobardt et al., 2007). Rather, given their high surface

expression, HIV-1 binding is probably mediated via non-canonical receptors such as GalCer and HSPG's (e.g. syndecans) but unlikely to be via DC-SIGN, which was expressed at very low levels. Indeed, one study indicated that HSPGs but not GalCer were the key moieties involved in HIV-1 binding to vaginal epithelial cells (Bobardt et al., 2007).

Our extensive binding data did not indicate an R5 or X4 preference in binding to A431 cells with both viruses apparently binding equally well. However, trypsin digestion experiments indicated that R5 virus predominantly utilized protein (e.g. HSPG) moieties to bind vaginal epithelial cells whereas X4 virus predominantly utilized non-protein (e.g. GalCer) moieties. Apart from binding GalCer, another potential reason for why X4 virus is more resistant to trypsin digestion is that X4 virus may somehow be protected by epithelial structures on the surface.

Whether viral genome integration occurs in vaginal epithelial cells is currently unclear, but this may be reflective of the different experimental procedures and cell types used in different studies. One study detected proviral HIV-1 DNA in between 29-68% vaginal and cervical clinical samples, indicating viral integration (Iversen et al., 1998). However, the samples may have included immune cells that were present in mucosa or secretions at the time of collection, especially since bleeding was observed in approximately 50% of patients upon cervical sample collection. Another study detected proviral HIV-1 DNA in three carcinoma cell lines (HEC1A, endometrium; CaSki, cervix; SiHa, uterus) and primary vaginal epithelial cells (Berlier et al., 2005). There was a preference for X4 integration, which was CXCR4 dependent since SDF-1, a CXCR4 ligand, blocked integration. X4 integration was probably the result of all three cell lines expressing high amounts of CXCR4 (~60%). Studies with primary gingival (oral) (Liu et al., 2003) and uterine (Asin et al., 2004) epithelial cells have also demonstrated preferential integration of X4 virus, but this may also have been due to high surface expression of CXCR4 and GalCer, which together can be used as alternative receptors to CD4 for viral entry (Liu et al., 2003). However, other studies have failed to observe HIV-1 *gag* DNA in primary cervical epithelial cells or the cervical ME-180 cell line (Dezzutti et al., 2001), or the ectocervix cell line (Ect1/E6E7) despite HIV-1 being

1 captured and subsequently released (Wu et al., 2003). The latter study further confirmed a lack of
2 viral integration using a luciferase reporter virus expressing CCR5 gp120 envelope (Wu et al.,
3 2003). Using a sensitive real-time PCR assay not used in any of the above studies we provide strong
4 evidence that both X4 and R5 virus are unable to integrate their genomes into the DNA of A431
5 vaginal epithelial cells. One explanation for this may be the very low surface expression levels of
6 CXCR4 in A431 cells (~5%). In this regard, the origin of A431 cells (vulval), which is different to
7 those used in the above studies, may account for the differences in CXCR4 expression and hence
8 X4 viral integration. Another explanation may be that, unlike the above studies, our samples were
9 rigorously treated with DNase and DpnI digestion to prevent possible false positive integration
10 events from contaminating HIV-1 DNA. Interestingly, another study showed that HIV-1 proviral
11 DNA could be detected in differentiated colonic epithelial cell clones but not undifferentiated
12 clones (Furuta et al., 1994). If applicable to vaginal epithelial cells, this may have implications for
13 HIV-1 transmission *in vivo*, as the virus may more readily integrate into apical (differentiated)
14 rather than basal (undifferentiated) epithelial cells. This may provide some explanation for why we
15 did not detect proviral DNA in A431 cells as they are an undifferentiated cell line (Knuechel et al.,
16 1990).

17 To further demonstrate if HIV-1 infection is supported in A431 vaginal epithelial cells we
18 employed three different approaches; infection with HIV-1 gp160 pseudotyped virus, detection of
19 spliced HIV-1 *tat* mRNA, and *de novo* production of p55 gag protein. However, we note that the
20 HIV-1 *tat* assay does not ascertain if entry or internalization of the virus occurred since the *tat*
21 mRNA PCR was not sensitive enough to detect the presence of low levels of spliced *tat* mRNA,
22 which can be expressed from non-integrative viral DNA (Sloan et al., 2010; Wu & Marsh, 2003).
23 Irrespective, we demonstrate that HIV-1 infection, *de novo* HIV-1 protein production and viral
24 assembly is not supported in A431 vaginal cells. These observations, together with the general
25 absence of CD4/CXCR4/CCR5 expression in A431 cells, support the view that productive HIV-1
26 infection requires canonical receptor expression on the host cell. Our findings are in concordance

1 with the majority of other studies demonstrating a lack of productive HIV-1 infection in vaginal
2 epithelial cells despite the presence of HSPGs and GalCer (Dezzutti et al., 2001; Greenhead et al.,
3 2000; Wu et al., 2003; Hu et al., 1998; Miller, 1998). However, they are in contrast with other
4 studies which indicate that vaginal epithelial cells can support productive HIV-1 infection (Phillips
5 et al., 1998; Furuta et al., 1994; Phillips et al., 1994; Tan et al., 1993). We have also demonstrated
6 that trypsin treatment failed to remove all surface-bound HIV-1. This raises an important issue in
7 other co-culture studies that have claimed infection of permissive cells as a result of *de novo* virus
8 production in epithelial cells. In some of these studies it is possible that new viral progeny may have
9 originated from trypsin-resistant bound HIV-1, which was transferred to the permissive cells from
10 the epithelial cell surface, leading to their infection.

11 Several studies have reported that HIV-1 may be sequestered in epithelial cytosolic endocytic
12 compartments or transmitted by transmigration/transcytosis (Hladik & McElrath, 2008; Hocini &
13 Bomsel, 1999; Hocini et al., 2001; Bobardt et al., 2007; Bomsel, 1997). Whether viral endocytosis
14 into epithelial cells results in productive infection is uncertain. Whilst one study showed that HIV-1
15 released by infected T-cells in vesicles were taken up by and productively infect cervical (ME100)
16 carcinoma epithelial cells (Tan et al., 1993), another study showed a lack of productive infection
17 after 18 days despite integrated proviral DNA being present (Berlier et al., 2005). To address
18 whether HIV-1 entry via endocytosis results in productive infection we utilized a GFP-encoding
19 VSV-G pseudotyped HIV-1 virus, which utilizes the endocytic pathway for cell entry and by-passes
20 conventional CD4 receptor-mediated entry mechanisms. This virus was able to establish a
21 productive infection in A431 cells that could be inhibited with AZT, demonstrating two key points.
22 First, HIV-1 does not normally enter epithelial cells via the endocytic pathway (otherwise a
23 productive infection would ensue with live virus) and that HIV-1 binding is probably mediated
24 through non-canonical viral-specific receptors. Second, epithelial cells are able to assemble and
25 secrete infectious viral progeny if receptor-mediated entry is by-passed (as VSV-G pseudotyped
26 HIV-1 virus establishes productive infection). Together with the fact that HIV-1 infection of TZM-

1 bl cells (epithelial-like cells engineered to express CD4, CXCR4 and CCR5) also results in the
2 assembly and secretion of infectious viral progeny, our data suggests that vaginal epithelial cells are
3 able to support productive viral infection only if HIV-1 gains entry into the cell through non-
4 conventional (endocytic) mechanisms. Our findings raise the intriguing possibility that if conditions
5 arise *in vivo* that enable receptor-mediated entry to be by-passed, for example during inflammatory
6 responses, productive HIV-1 infection may be supported in vaginal epithelial cells.

7 We propose that under 'normal' conditions productive HIV-1 infection is unlikely to occur in
8 vaginal epithelial cells. However, post-capture, infectious virus may remain immobilized on the
9 surface giving rise to the possibility of transmission to permissive cells in the underlying mucosa. A
10 number of studies support this hypothesis (Yeaman et al., 1998; Shen et al., 2011; Ballweber et al.,
11 2011; Kaldensjo et al., 2011; Shen et al., 2009; Miller & Shattock, 2003; Khanna et al., 2002;
12 Bhoopat et al., 2001; Hu et al., 2000; Hu et al., 1998; Spira et al., 1996; Dezzutti et al., 2001;
13 Howell et al., 1997; Wu et al., 2003), although others could not demonstrate viral transfer to
14 permissive cells (PBMCs) by cell-cell contact (Berlier et al., 2005). Utilizing a cell overlay
15 experiment we show that HIV-1 retains infectivity on the A431 epithelial cell surface and can be
16 readily transferred to permissive TZM-bl cells to establish a productive infection. Although we are
17 uncertain of the surface moieties that enable binding and transfer, others have demonstrated an
18 important role for HSPGs, since heparin or heparin sulfate can inhibit gp120 binding to CD4 cells
19 and heparinase treatment can reduce viral attachment (Mondor et al., 1998; Roderiquez et al., 1995;
20 Bobardt et al., 2003; Wu et al., 2003). These findings have implications for HIV-1 infection *in vivo*
21 as this may provide a window of opportunity for infectious immobilized virus to be transferred to
22 susceptible immune cells in the sub-mucosa, thereby establishing an acute infection and
23 disseminating the virus in the body. This is supported by both macaque and human studies, which
24 indicate that viral transmission is facilitated by the presence of HIV-1 target cells (dendritic cells,
25 Langerhans cells, CD4⁺ T cells and macrophages) in the uterus, endocervix, ectocervix and vagina
26 (Yeaman et al., 1998; Shen et al., 2011; Ballweber et al., 2011; Kaldensjo et al., 2011; Shen et al.,

2009; Miller & Shattock, 2003; Khanna et al., 2002; Bhoopat et al., 2001; Hu et al., 2000; Hu et al., 1998; Spira et al., 1996; Maher et al., 2005; Hu et al., 2004; Greenhead et al., 2000; Hladik et al., 2007; Zhang et al., 1999; Asin et al., 2004).

In summary, although we caution that our experiments have been performed with a carcinoma cell line, our data conform to primary cell studies and support the view that HIV-1 is readily captured by vaginal epithelial cells (probably via non-canonical viral-specific receptors) but that genome integration and productive viral infection does not occur. However, vaginal epithelial cells possess the cellular machinery to support productive HIV-1 infection if the virus enters via the endocytic pathway or if conventional entry mechanisms are by-passed. Once captured, HIV-1 also remains infectious on the surface of epithelial cells, which may facilitate direct viral transfer to permissive cells in the sub-mucosa thereby establishing acute infection.

Methods

Cell lines, primary cells, viruses and virus-like particles

The A431 cell line (epidermoid carcinoma of vulval origin) (Giard et al., 1973) and 293T (renal epithelial) cells were obtained from the American Type Culture Collection. Human glioma cells (NP2) expressing human CD4 and CXCR4 or CCR5 have been previously described (Soda et al., 1999). The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl cells (catalogue no. 8129), PM1 cells (catalogue no. 3038), C8166 T cells (catalogue no. 404 donated by Robert Gallo), JTLRG-R5 (catalogue no. 11586) and HIV-1 molecular clones pYU2 (R5-utilizing, catalogue no.1350) and pLAI.2 (X4-utilizing, catalogue no. 2532). The HIV-1 *gag-pol* expression vector p8.91 and the vesicular stomatitis virus envelope protein (VSV-G) expression vector pMDG were kindly provided by Didier Trono (University of Geneva, Switzerland). The HIV gp160 envelope vectors (pHXB2 (X4), pYU2 (R5) and pSVIII 89.6 (dual tropic)) were a gift from Professor Greg Towers, University College London. The retroviral packaging vector pCSGW encoding GFP was a kind gift from

Adrian Thrasher, Institute of Child Health, University College London, UK. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM, PAA) supplemented with 10% fetal bovine serum (FBS) (PAA), 100 U of penicillin per mL, and 100 µg of streptomycin per mL (PAA) at 37°C and 5% CO₂. NP2 cells were additionally supplemented with 1 mg/mL of G418 (Invitrogen) and 1 µg/mL of puromycin (Invitrogen).

Virus preparation

Viral vectors used for production of VSV-G and HIV gp160 pseudotyped HIV were prepared by transient transfection of 293T cells using a protocol adapted from Besnier *et al.* (Besnier et al., 2002). Briefly, 293T cells were seeded at 95% confluency in a 10 cm dish and the following day cells were transfected with 3 µg each of pMDG and p8.91 and 4.5 µg of pCSGW using the polyanionic transfection reagent Jet PEI (Polyplus Transfection) according to the manufacturer's instructions. After 24 h the media was replaced and 48, 72 and 96 h post-transfection virion-containing culture supernatants were harvested and filtrated through a 0.45µm pore size membrane and stored in aliquots at -80°C until required. Production of infectious stocks of live virus was performed by transient transfection of 293T cells as described above, with 5 µg of the infectious molecular clone pLAI.2 (X4) and or pYU2 (R5) used per transfection. For trypsin sensitivity experiments and detection of integration into epithelium, YU2 virus was grown in NP2-R5 and JLTRG-R5 cells while LAI virus was grown in C8166 cells for 1-2 weeks with addition of fresh medium until cells showed accumulation of cytopathic effects and were then harvested and frozen in aliquots.

Virus titration

Infectious virus stock (LAI and YU2) titers were determined by plaque assay. Briefly, TZM-bl cells (1×10^4 cells/well) were cultured overnight (96-well plates) and incubated with eight replicates of ten serial dilutions (0.5 log) of virus stock in a total of 100 µL growth media per well. After 48 h,

1 virus supernatant was removed and the cells were fixed with 0.05% glutaraldehyde for 5 min at
2 room temperature and washed twice with phosphate-buffered saline (PBS). Expression of β -
3 galactosidase was determined by staining cells with X-Gal stain [1mg/mL X-Gal in 5 mM
4 $\text{KFe}_4(\text{CN}_6) \cdot 3\text{H}_2\text{O}$, 5 mM $\text{KFe}_3(\text{CN}_6) \cdot 3\text{H}_2\text{O}$, and 1 mM MgCl_2] and incubating culture plates at 37°C
5 for 2 h. Virus infectivity was estimated as plaque forming units (PFU) per mL. Titration of VSV-G
6 pseudotyped HIV-1 and HIV gp160 pseudotyped HIV-1 was carried out using 293T cells or NP2
7 cells, respectively. Cells were seeded at 1×10^5 cells/well (24 well plates) and cultured overnight at
8 37°C. Serial dilutions (1:2) of virus supernatant were applied to the cells (500 μL) and incubated
9 overnight. The following day the media was exchanged and 48 h after transduction with virion-
10 containing culture supernatants the percentage of GFP-expressing cells was determined by flow
11 cytometry using the FACSCanto machine (BD Biosciences). Data was analyzed with FACSDiva
12 software and WinMDI (copyright 1993-2000 Joseph Trotter <http://facs.scripps.edu>) to calculate the
13 infectious units per mL.

15 **HIV-1 receptor expression by quantitative reverse transcription-PCR**

16 RNA was isolated from resting TZM-bl and A431 cells using GenElute Mammalian Total RNA
17 Miniprep Kit (Sigma), followed by treatment with Turbo DNA free DNase (Ambion) according to
18 the manufacturer's instructions. All samples were confirmed DNA free prior to analysis. cDNA was
19 synthesized from 1 μg of RNA using HIV reverse transcriptase (Ambion) according to the
20 manufacturer's instructions. Primers were obtained from RTPPrimerDB
21 (<http://medgen.ugent.be/rtprimerdb/>) and PrimerBank (<http://pga.mgh.harvard.edu/primerbank>)
22 (Lefever et al., 2009; Spandidos et al., 2010). Gene expression of CD4, CCR5, CXCR4, DC-SIGN,
23 SDC-1 (syndecan-1) and SDC-4 (syndecan-4) was quantified by real-time PCR using SYBR Green
24 JumpStart Taq Ready Mix (Sigma) with 4 pmol primers and 1 μL cDNA in 10 μL reactions on the
25 Corbett Research Rotor-Gene 6000 (Qiagen) using the following cycling parameters: 95°C for 3
26 min; followed by 95°C for 3 s, annealing for 10 s and extension for 20-30 s for 40 cycles. Data was

analyzed with Corbett Research Rotor-Gene 6000 Series Software 1.7 using the two standard curve method with β -actin used as the normalizer gene. Primer sequences, annealing and extension temperatures are listed in Table 1.

HIV-1 receptor expression by flow cytometry

A431 and TZM-bl resting cells were washed with PBS and incubated with 0.02% (W/V) EDTA for 5-30 min. Detached cells were washed thoroughly with PBS supplemented with 1% BSA and 0.01% azide (wash buffer), and resuspended at 1×10^6 cells in 1 mL wash buffer. To identify surface expressed HIV-1 receptors and co-receptors, 100 μ L of cells were incubated at room temperature for 1 h with mouse anti-human CD4 (1:4 catalogue no.724), CCR5 (1:20, catalogue no. 4090), CXCR4 (1:80, catalogue no. 4083), DC-SIGN (1:100 catalogue no. 6884) monoclonal antibodies (all obtained from the AIDS Research and Reference Reagent Program), GalCer (1:200, anti-galactocerebroside, Millipore) or heparan sulfate proteoglycan (1:200, Millipore) monoclonal antibodies. Primary antibodies were detected with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch). After thorough washing, cells were fixed in 200 μ L 4% formaldehyde and the percentage of FITC-expressing cells was determined by flow cytometry.

Detection of HIV-1 binding and replication by Western Blot

A431 and TZM-bl cells were seeded at 5×10^5 cells per well and the following day infected with YU2 (R5) or LAI (X4) virus at a multiplicity of infection (MOI) of 0.2. After overnight incubation at 37°C the cells were washed to remove unbound virus. Cells were harvested in 250 μ L 1 x RIPA buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 20mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS, supplemented with Halt complete protease inhibitor cocktail (Perbio Science)], placed on ice for 30 min, and stored at -80°C until required. Total protein lysates (mammalian and viral) were normalized for protein content using the bicinchoninic acid (BCA)

assay (Pierce) and separated using 12% SDS-PAGE gels. Proteins were transferred to PVDF membranes, probed with anti-HIV-1 gag monoclonal antibody recognizing p24 and p55 isoforms (catalogue no.6457; AIDS Research and Reference Reagent Program) and secondary goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated antibody (Jackson ImmunoResearch), before developing using Immobilon-ECL (Millipore). α -actin was used as a loading control.

Detection of HIV-1 binding and packaged viral RNA by PCR

A431 and TZM-bl cells were seeded at 5×10^5 cells per well and the following day infected with YU2 (R5) virus at an MOI of 0.2. After overnight incubation at 37°C the cells were washed to remove unbound virus. Total RNA was isolated as above and confirmed DNA free prior to analysis. Equal amounts of total RNA was used to detect packaged viral RNA by first synthesizing cDNA using Superscript cDNA Synthesis Kit (Invitrogen) and an HIV-1 specific primer (5'-GTC ATG AAA CAA ACT TGG C-3'). A 2 μ L aliquot of cDNA was then subjected to nested PCR using primers to amplify a 2 kb fragment of the HIV *pol* gene. First round PCR was performed in a 20 μ L reaction containing 1 x PCR buffer, 100 μ M dNTP's, 1.5 mM MgSO₄, 2.5 U *Taq* polymerase (New England Biolabs) and 10 pmol of each primer (Forward: 5'-AAT GAT GAC AGC ATG TCA GGG AGT-3'; Reverse: 5'-AGT CTT TCC CCA TAT TAC TAT GCT TTC-3'). Cycle parameters were as follows: 95°C for 5 min; 94°C for 10 s, 55°C for 30 s, and 72°C for 1 min for 30 cycles; and an extension of 72°C for 10 min. For subsequent nested PCR, 1 μ L of the first round PCR reaction was used as a template to amplify an internal region of the *pol* gene and was performed in a 10 μ L reaction containing 1 x SYBR Green JumpStart Taq Ready Mix (Sigma), and 3 pmol of each primer (Forward: 5'-TTC TTC AGA GCA GAC CAG-3'; Reverse: 5'-ACT TTT GGG CCA TCC ATT-3'). Cycle parameters were 95°C for 3 min; followed by 95°C for 1 min, 55°C for 30 s, and 72°C for 1 min for 35 cycles; and an extension of 72°C for 10 min. PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

1 **Whole virus binding and trypsin sensitivity**

2 A431 cells (5×10^4) were infected with either YU2 (R5) or LAI (X4) virus at an MOI of 5
3 overnight at 4°C. Cells were washed three times with PBS and blocked in PBS/10% BSA for 10
4 min at room temperature. To determine whether HIV-1 binding was trypsin sensitive, prior to
5 blocking cells were treated with trypsin (0.05%)-EDTA (0.02%) (PAA, UK) for 5 min at 37°C.
6 Cells were gently removed by scraping and labeled with HIV-1 gp120 monoclonal antibody F425
7 B4e8 (1:200) (AIDS Research and Reference Reagent Program) followed by Cy5-conjugated
8 AffinityPure goat anti-human IgG secondary (1:400) (Jackson ImmunoResearch), each for 30 min
9 at 4°C. Cells were washed three times with PBS, resuspended in 4% formaldehyde and subjected to
10 flow cytometry. Binding percentages for whole virus were calculated as increased Cy5 shift of
11 HIV-1 infected labelled cells from uninfected labelled cells. Given the higher background with a
12 new batch of antibody, binding percentages for the trypsin sensitivity data were calculated as
13 increased Cy5 shift relative to HIV-1 infected secondary alone labelled cells.

14 15 **Productive viral infection by detection of spliced HIV-1 *tat* by PCR**

16 A431 and TZM-bl cells were seeded at 5×10^5 cells per well and the following day infected with
17 YU2 (R5) or LAI (X4) virus at an MOI of 0.2. After overnight incubation at 37°C unbound virus
18 was removed by washing and total RNA isolated (GenElute Mammalian Total RNA Miniprep Kit;
19 Sigma). Genomic DNA was removed with Turbo DNase free (Ambion) according to the
20 manufacturer's instructions and samples were confirmed DNA free prior to analysis. Equal amounts
21 of total RNA was used to synthesize viral cDNA transcripts using the HIV-specific oligo ART-7 5'-
22 TTC TAT TCC TTC GGG CCT GTC G -3'. A 1 µL aliquot of cDNA was then subjected to PCR
23 using primers spanning the *tat*1 and 2 exon junctions (*tat*-junction forward: 5'- TAG ATC CTA
24 GAC TAG AGC CC-3' and *tat*-junction reverse 5'- TTG GGA GGT GGG TCT GAA ACG-3') in a
25 20 µL reaction containing 1 x PCR buffer, 100 µM dNTP's, 1.5 mM MgSO₄, 2.5 U *Taq* polymerase
26 (New England Biolabs) and 10 pmol of each primer. Cycle parameters were as follows: 95°C for 5

min; followed by 94°C for 10 s, 55°C for 30 s, and 72°C for 1 min for 35 cycles; with a final extension of 72°C for 10 min. PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

Detection of HIV-1 integration by primer-probe Alu-LTR PCR assay

To determine whether HIV DNA was able to integrate into epithelial cells a real-time PCR assay was performed with HIV-1 LTR and human Alu-specific primers with a U5 specific probe as previously described (Mbisa et al., 2009). In this protocol the forward primer binds to the U5 region of the HIV-1 LTR, the reverse primer binds to the *Alu* repeat sequence in the host genome, and the internal FAM-TAMRA labelled probe binds in between these regions (but also binds the U5 region of the HIV-1 LTR). The specific utilisation of the U5 specific FAM-TAMRA probe also adds specificity to assay. During PCR, this assay amplifies the majority of the HIV-1 LTR together with some host genomic material, and a PCR product can only be amplified if the HIV-1 LTR has integrated into the host genome. A431 and PM-1 (control) cells were seeded at 5×10^5 cells per well and the following day infected with YU2 (R5) or LAI (X4) virus, pre-treated with RNase-free DNase (Roche, UK) at 37°C for 1 h with 4mM MgCl₂. MOI's ranged from 1 to 140. Heat-inactivated virus (60°C for 1 h) without DNase treatment was used as a DNA contamination control. Cells and virus were incubated for 48 h at 37°C, after which cells were washed three times with PBS before DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, Poole, UK) according to the manufacturer's instructions with Proteinase K digestion for 20 min. Isolated DNA samples were digested with DpnI (New England Biolabs, UK) to degrade any plasmid DNA contaminant. DNA was then quantified by Nanodrop and either 50 ng or 100 ng DNA was analysed by real-time PCR on a Rotorgene 6000 (Qiagen, UK) using primers 0.2 M MH535 forward (5'-AACTAGGGAACCCACTGCTTAAG-3') and 0.8 M reverse SB704 (5'-TGCTGGGATTACAGGCGTGAG-3') with 0.2 M probe P-HUS-SS1 (5'-FAM-TAGTGTGTGCCCCGTCTGTTGTGTGAC-TAMRA-3') using Jumpstart Ready Mix (Sigma,

Poole, UK) in 10 μ L reactions. For each PCR reaction the same concentration of DNA was used for all samples isolated from individual cell lines. Samples were denatured for 10 min followed by 60 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 60 s. DNase-treated virus exposed samples were compared with heat-inactivated virus exposed samples. Positive integration events were taken as a lower C_t in the DNase treated virus exposed sample than the heat-inactivated virus control.

HIV-1 integration and productive infection using pseudotyped virus-like particles

A431, NP2-R5 and NP2-X4 cells were seeded at 1×10^5 cells per well and cultured overnight at 37°C. Serial dilutions (1:2) of HIV-1 gp160 pseudotyped (X4, R5 and dual tropic) and VSV-G pseudotyped HIV-1 were applied and incubated overnight at 37°C. HIV-1 integration and *de novo* virus protein production were determined by the presence of GFP-expressing cells by flow cytometry. To inhibit HIV-1 specific GFP expression, infections were also carried out in the presence of 500 μ M of the HIV-1 reverse transcriptase inhibitor AZT (NIH AIDS Reagent Program Cat no.3485).

Detection of *de novo* HIV-1 production by indicator cell infection

A431 and TZM-bl cells were seeded at 5×10^5 cells per well and the following day infected with YU2 (R5) or LAI (X4) virus at an MOI of 0.2. After overnight incubation at 37°C the cells were extensively washed with HBSS (Invitrogen) to remove unbound virus. Fresh media was applied to the cells and the plates were incubated at 37°C for up to 7 days to allow any *de novo*-produced infectious virus to be released into the medium. Culture medium (potentially containing infectious virus) was then applied to 3×10^5 TZM-bl indicator cells and incubated for a further 24 h at 37°C. Cells were fixed, washed twice with PBS and stained for β -galactosidase expression with X-Gal stain. Individual wells were visualized by light microscopy at 100 X magnification.

HIV-1 transfer assay

A431 and TZM-bl cells were seeded at 1×10^5 cells and the following day infected with YU2 (R5) or LAI (X4) virus at an MOI of 0.2. After overnight incubation at 37°C the cells were thoroughly washed in HBSS to remove any unbound virus. Controls included A431 cells without the addition of virus. TZM-bl cells (3×10^5) were then overlaid onto the epithelial cells and the plates incubated for a further 48 h at 37°C. Cells were fixed and stained for β -galactosidase expression with X-Gal stain. Individual wells were photographed by light microscopy at 100 X magnification.

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1 **Figure Legends**

2 **Figure 1.** Basal HIV-1 receptor mRNA expression in resting vaginal epithelial cells. A431 and
3 TZM-bl cells were examined for mRNA expression of CD4, CCR5, CXCR4, DC-SIGN and the
4 HSPG's syndecan-1 and -4 by quantitative RT-PCR. Data are presented as mRNA transcripts
5 (arbitrary units) normalized to β -actin in a minimum of three independent experiments. Bars
6 indicate \pm standard deviation from the mean.

7

8 **Figure 2.** Basal HIV-1 receptor surface expression in resting vaginal epithelial cells. A431 and
9 TZM-bl cells were examined for surface expression of CD4, CCR5, CXCR4, DC-SIGN, GalCer
10 and HSPG's by flow cytometry using monoclonal primary antibodies specific to each receptor with
11 a FITC-labeled secondary antibody. Data are presented as percentage of cells expressing each
12 receptor in a minimum of three independent experiments. Bars indicate \pm standard error of the
13 mean.

14

15 **Figure 3.** HIV-1 R5 and X4 binding to vaginal epithelial cells. A431 and TZM-bl cells were
16 incubated with cell free YU2 (R5) or LAI (X4) infectious virus overnight under identical conditions
17 and extensively washed. (A) Detection of p24 gag protein by Western blotting using α -actin as a
18 loading control; (B) Detection of immobilized virus on the cell surface by flow cytometry. Bars
19 indicate \pm standard deviation from the mean; (C) Detection of packaged HIV R5 RNA by
20 amplification of the HIV-1 *pol* gene using nested PCR. (D) Percentage reduction in detection of
21 immobilized virus on the cell surface by flow cytometry after trypsin treatment. Virally exposed
22 cells are compared with cells labeled with secondary alone. Data sets (A-D) are representative of
23 three or four independent experiments.

24

25 **Figure 4.** Post-integration HIV-1 mRNA transcription and *de novo* viral protein production in
26 vaginal epithelial cells. (A) Detection of spliced HIV-1 *tat* mRNA in A431 and TZM-bl control

cells by PCR 24 h post-infection with YU2 (R5) or LAI (X4) infectious virus. (B) p55 gag protein detection in A431 and TZM-bl control cells by Western blot after 24 h infection with R5 (YU2) and LAI (X4) virus. (C) Infection of A431 and NP2-R5/X4 control cells with GFP-linked single-cycle X4, R5 and dual tropic HIV-1 gp160 pseudotyped virus and detection of GFP incorporation into epithelial cell DNA by flow cytometry. Data are representative of three independent experiments.

Figure 5. HIV-1 entry via the endocytic pathway results in productive viral infection in vaginal epithelial cells. Two fold serial dilutions of VSV-G pseudotyped HIV-1 (MOI 1 – 0.125) were added to A431 and TZM-bl control cells. Infection is measured by flow cytometry as % GFP expression (black bars). The effect of AZT (500mM) on GFP expression was also measured at the highest virus inoculum (white bar). Data are representative of three independent experiments

Figure 6. Transfer of captured HIV-1 from vaginal epithelial cells to permissive cells. A431 and TZM-bl cells were incubated with R5 (YU2) and LAI (X4) virus for 24 h and following extensive washing TZM-bl indicator cells were added for a further 48 h. Controls included A431 cells without the addition of virus. Data are representative of three independent experiments.

1 **Table 1. Primer sets detecting gene expression of HIV-1 associated receptors**

2

Gene	Primer	Sequence	Annealing temp. (°C)	Extension temp. (°C)	Product (bp)
CD4	Forward	5'- ACTAAAGGTCCATCCAAGCTGA —3'	60	75	151
	Reverse	5'- GCAGTCAATCCGAACACTAGCA —3'			
CCR5	Forward	5'- TGGACCAAGCTATGCAGGTG —3'	58	75	240
	Reverse	5'- CGTGTCACAAGCCCACAGAT —3'			
CXCR4	Forward	5'- CCTCATCCTGGCTTTCTTCG —3'	60	75	285
	Reverse	5'- GAATGTCCACCTCGCTTTCC —3'			
DC-SIGN	Forward	5'- TCAAGCAGTATTGGAACAGAGGA —3'	60	75	136
	Reverse	5'- CAGGAGGCTGCGGACTTTTT —3'			
Syndecan-1	Forward	5'- TGAAACCTCGGGGAGAATAC —3'	60	75	171
	Reverse	5'- GGTACAGCATGAAACCCACC —3'			
Syndecan-4	Forward	5'- CAGGGTCTGGGAGCCAAGT —3'	58	72	129
	Reverse	5'- GCACAGTGCTGGACATTGACA —3'			
β-actin	Forward	5'- CATGTACGTTGCTATCCAGGC —3'	58	75	250
	Reverse	5'- CTCCTTAATGTCACGCACGAT —3'			

3

4

5

Table 2. Detection of integrated HIV-1 genome in A431 vaginal epithelial cells

Cell line	R5-YU2 (MOI = 7.5)	X4-LAI (MOI = 10)	X4-LAI (MOI = 140)
A431	ND	ND	ND
C8166 ^a	n/a	+ (Ct = 33)	+ (Ct = 34)
NP2-R5 ^b	+ (Ct = 29)	n/a	n/a

^a C8166 cells express CXCR4 and were used for X4 viral infections only.

^b NP2-R5 cells express CCR5 and were used for R5 viral infections only.

+, Integrated HIV-1 product detected (cycle threshold detection in brackets).

ND, Integrated HIV-1 product not detected.

n/a, not applicable

Figure 1
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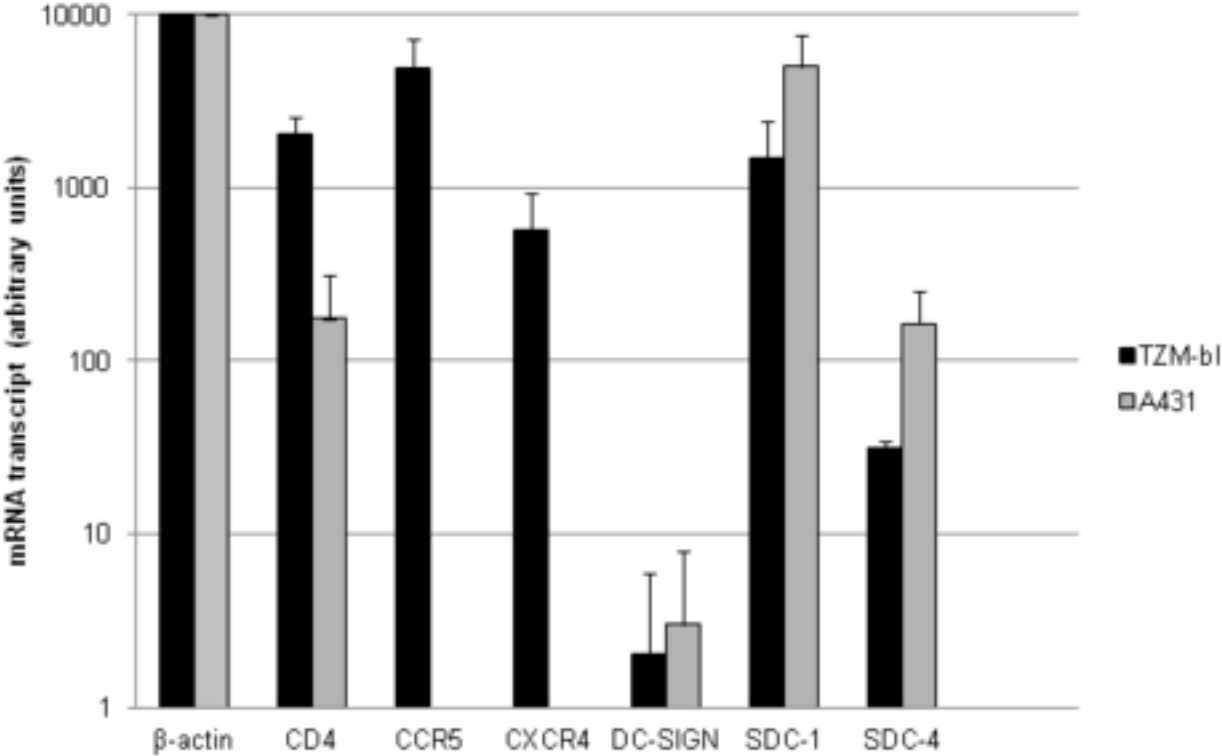


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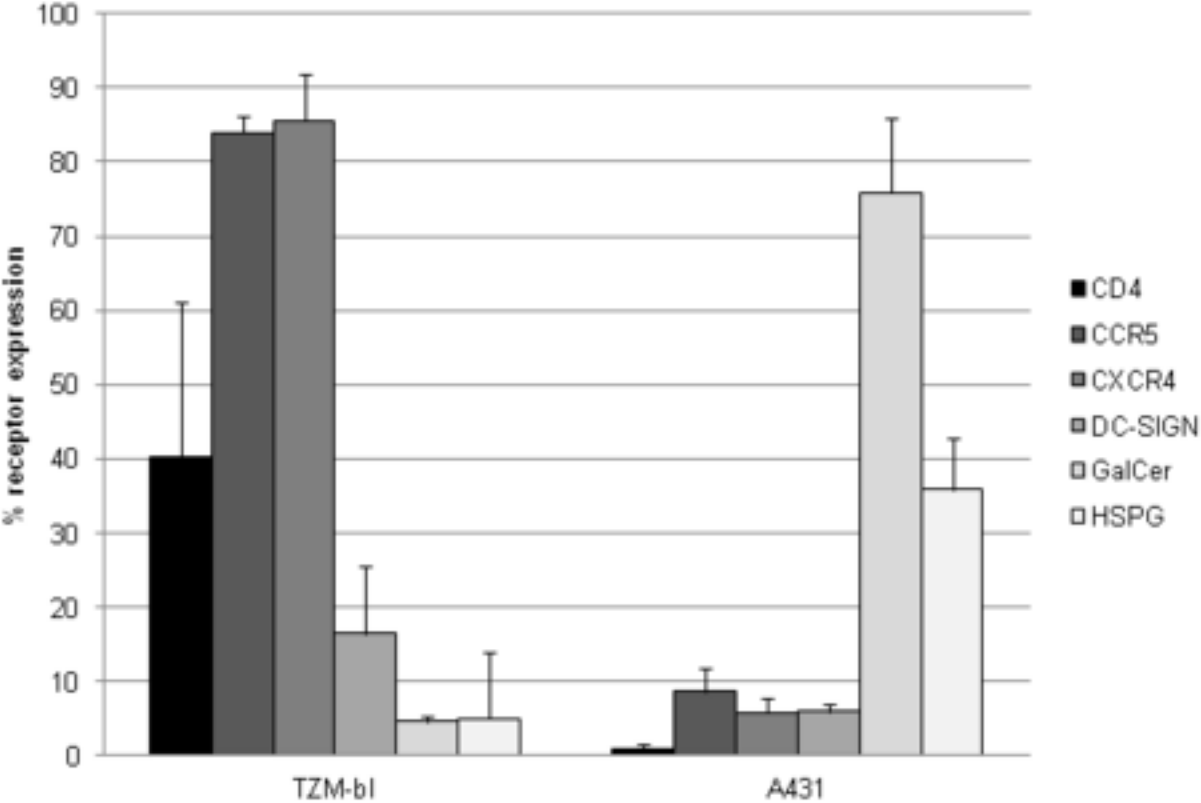


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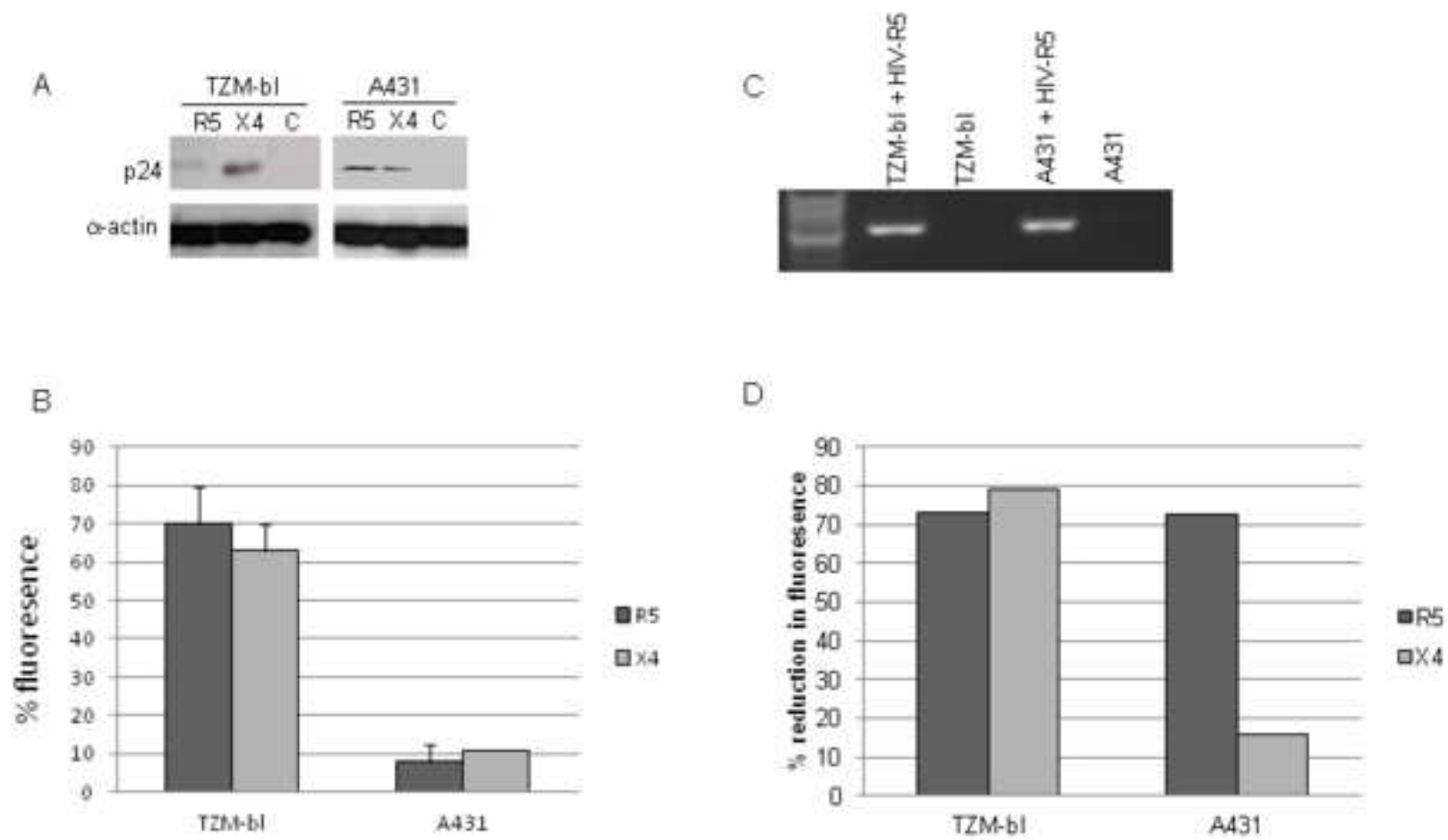


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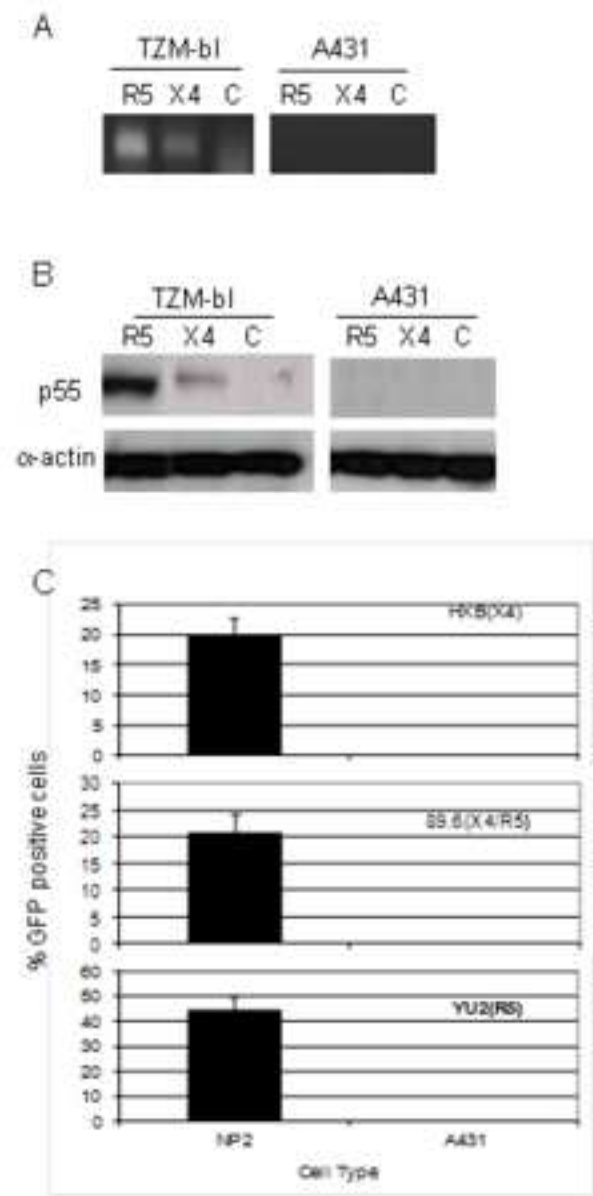


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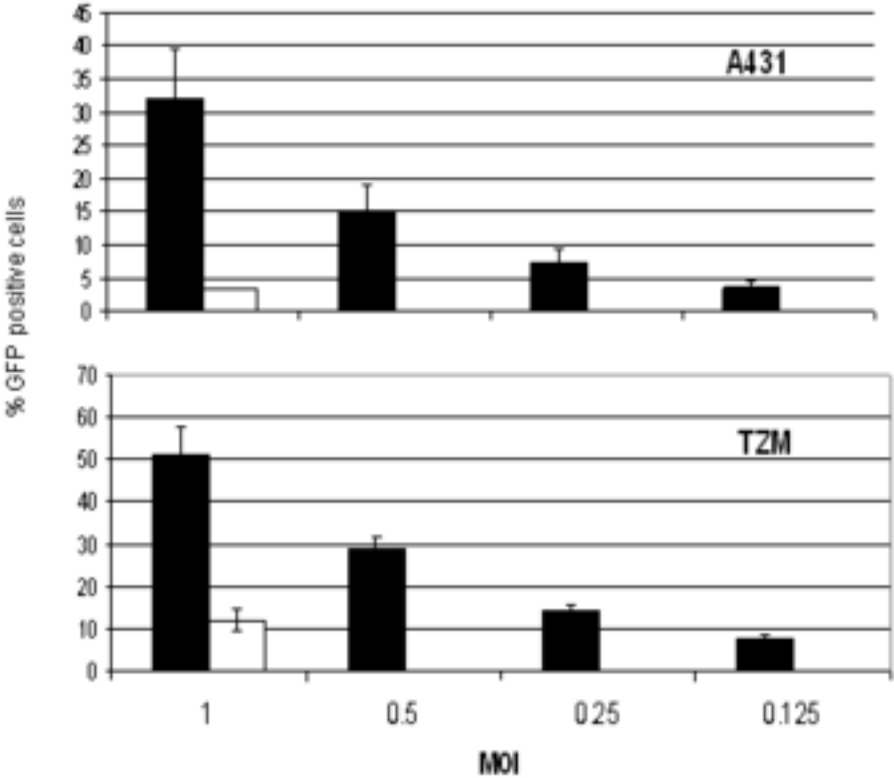


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